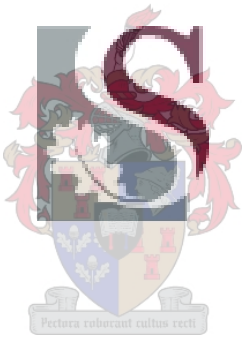
Investigation of resveratrol production by genetically engineered *Saccharomyces cerevisiae* strains

by

Kim Trollope



Thesis presented in partial fulfilment of the requirements for the degree of Master of Sciences at Stellenbosch University.

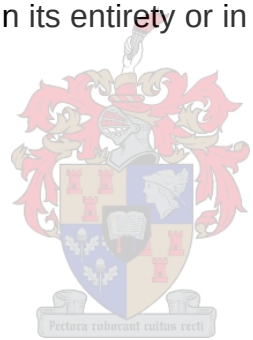
December 2006

Supervisor:
Maret du Toit

Co-supervisor:
Melané Vivier

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.



_____ _____

Kim Trollope **Date**

This thesis is dedicated to my family Winston, Lynne, Penelope and Brett.
Hierdie tesis is opgedra aan my familie Winston, Lynne, Penelope and Brett.

BIOGRAPHICAL SKETCH

Kim Trollope was born in Alice, South Africa, on 9 May 1977. She attended primary school in Fort Beaufort and matriculated from Victoria Girls' High School, Grahamstown. Kim obtained her BSc degree from Stellenbosch University in 2002. In 2003 she received the degree BScHons from the Institute for Wine Biotechnology, Stellenbosch University. In 2004 she enrolled for the MSc degree at the same institute.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

DR MARET DU TOIT, Institute for Wine Biotechnology, Stellenbosch University who acted as my supervisor and provided guidance, support and encouragement during my studies;

PROF MELANÉ VIVIER, Institute for Wine Biotechnology, Stellenbosch University who acted as co-supervisor, and provided valuable scientific input and support;

DR MARIETJIE STANDER, Central Analytical Facility, Stellenbosch University for encouragement and valuable scientific contributions;

RESEARCH COLLEAGUES for their scientific discussions and advice with regards to practical work;

The **STAFF** at the Institute for Wine Biotechnology for their assistance and

The **NATIONAL RESEARCH FOUNDATION, WINETECH, THRIP** and **STELLENBOSCH UNIVERSITY** for financial support.

PREFACE

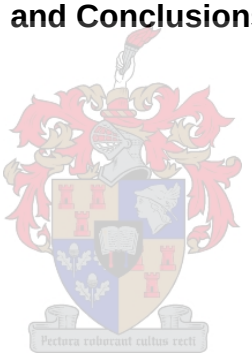
This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal, *Yeast,* to which Chapter 3 will be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Resveratrol – linking plants, yeasts and humans

Chapter 3 **Research Results**
Optimisation of both resveratrol production in recombinant *Saccharomyces cerevisiae* and the required analytical tools

Chapter 4 **General Discussion and Conclusions**



SUMMARY

Resveratrol is a phytoalexin that is produced in the leaves and skins of grape berries in response to biotic and abiotic factors. Substitution and polymerisation of resveratrol units produce an array of compounds which form part of the active disease defence mechanism in grapevine.

Wine is one of the major sources of resveratrol in the human diet. Resveratrol is one of the phenolic compounds present in wine that mediates protective effects on human health. It has been shown to prevent the development of cardiovascular disease, cancer and pathogenesis related to inflammation.

Red wines contain higher levels of resveratrol than white wines owing to extended maceration times during fermentation on the skins. During white wine vinification skin contact is limited as skins are removed prior to fermentation. Thus, the extraction of resveratrol into white wines is minimal. The principal focus of our research is the development of a wine yeast strain capable of resveratrol production during grape must fermentation. It is proposed that red and white wines produced with such a resveratrol-producing yeast will contain elevated levels of resveratrol, and that added health benefits may be derived from their consumption.

Initial work done in our laboratory established that expressing multiple copies of the genes encoding coenzyme A ligase (*4CL216*) and resveratrol synthase (*vst1*) in laboratory yeast enabled the yeast to produce resveratrol, conditional to the supplementation of the growth medium with *p*-coumaric acid. This study focused on the optimisation of resveratrol production in *Saccharomyces cerevisiae*. It involved the integration and constitutive expression of *4CL216* from hybrid poplar and *vst1* from grapevine. Integration and expression of these genes in three laboratory strains was confirmed by Southern and Northern blot analyses.

The evaluation of resveratrol production by yeast required the initial optimisation of the analytical techniques. We optimised the method for sample preparation from the intracellular fraction of yeast and devised a procedure for the assay of the extracellular fractions. The LCMSMS method was further developed to encompass detection and quantification of other compounds related to resveratrol production in yeast.

Comparison of resveratrol production in three different yeast genetic backgrounds indicated that the onset of production and the resveratrol yield is yeast strain dependent. Precursor feeding studies indicated that *p*-coumaric acid availability was a factor limiting maximal resveratrol production. Early indications were obtained that endogenously-produced resveratrol may have an impact on yeast viability during extended culture periods.

This study has broadened our understanding of the resveratrol production dynamics in *S. cerevisiae* and provided important indications as to where further optimisation would be beneficial in order to optimally engineer a wine yeast for maximal resveratrol production.

OPSOMMING

Resveratrol is ‘n fitoaleksien wat in die blare and doppe van druiwekorrels geproduseer word as gevolg van biotiese en abiotiese faktore wat op die wingerdplant inwerk. Substitusie en polimerisasie van resveratroleenhede het ‘n verskeidenheid verbindings tot gevolg, wat ‘n belangrike deel van die aktiewe weerstandsmeganisme in wingerd vorm.

Wyn is van die belangrikste resveratrolbronne in die menslike dieet. Resveratrol is een van ’n verskeidenheid fenoliese verbindings wat in wyn voorkom wat ‘n positiewe invloed op die mens se gesondheid het. Wat dit aanbetref, is daar al bewys dat dit die ontwikkeling van kardiovaskulêre siektes, kanker en patogenese wat met inflammasie verbind word, kan voorkom.

Rooiwyne se resveratrolvlakke is hoër as dié van witwyne as gevolg van die verlengde dopkontakperiodes gedurende die fermentasie van rooiwyne. Gedurende die maak van witwyne word die doppe egter voor fermentasie verwyder en die ekstraksie van resveratrol is dus minimaal in dié wyne. Die hoofdoel van hierdie navorsing is die ontwikkeling van ‘n wyngis wat resveratrol gedurende fermentasie van mos kan produseer. Die navorsing is gebaseer op die veronderstelling dat beide rooi- en witwyne wat met só ‘n resveratrolproduserende gis geproduseer is, verhoogde vlakke van resveratrol sal bevat en dat dit dus ook bykomende gesondheidsvoordele vir die verbruiker sal inhou.

Vorige navorsing in ons labarotorium het bewys dat laboratoriumgis oor die vermoë beskik om resveratrol te produseer wanneer die gene wat die koënsiem A ligase (*4CL216*) en resveratrolsintase (*vst1*) enkodeer op multikopie-plasmiede uitgedruk word indien die groeimedia met *p*-koumariensuur aangevul word. Hierdie studie het op die optimisering van resveratrolproduksie in *Saccharomyces cerevisiae* gefokus. Dit het die integrasie en konstitutiewe uitdrukking van *4CL216* uit hibriede populier en *vst1* uit wingerd behels, en die integrasie en uitdrukking van die gene in drie laboratoriumgiste is deur middel van Southern- en Northern-kladtegnieke bevestig.

Die evaluering van resveratrolproduksie deur gis het dit noodsaaklik gemaak dat die analitiese tegnieke aanvanklik geoptimiseer moes word. Dit is bereik deur middel van monstervoorbereiding vanuit die intrasellulêre fraksie van gis en ‘n prosedure wat uitgewerk is vir die bepaling van resveratrol in die ekstrasellulêre fraksie. Die LCMSMS-metode is verder ontwikkel om die opspoor en kwantifisering van verwante verbindings met betrekking tot resveratrolproduksie in gis ook moontlik te maak.

Vergelyking van resveratrolproduksie in drie giste van verskillende genetiese agtergronde het aangedui dat die begin van produksie en die resveratrolopbrengs is gisras-afhanklik. Voorlopervoerstudies het aangedui dat die beskikbaarheid van *p*-koumariensuur ‘n faktor was wat maksimale resveratrolproduksie beperk het. Vroeë aanduidings het getoon dat endogeen-geproduseerde resveratrol moontlik ‘n impak op gis se lewensvatbaarheid gedurende verlengde groeiperiodes kan hê.

Met hierdie studie is die kennis van die dinamiek van resveratrolproduksie in *S. cerevisiae* uitgebrei en dit het ook belangrike aanduiders verskaf watter aspekte geoptimiseer moet word om ‘n wyngis vir maksimale resveratrolproduksie optimaal geneties te verbeter.

CONTENTS

GENERAL INTRODUCTION AND SPECIFIC PROJECT AIMS

1.1 INTRODUCTION

Resveratrol is one of the phenolic compounds present in wine which is thought to be, in addition to ethanol, responsible for the health promoting effects of moderate wine consumption (Siemann and Creasy, 1992). Epidemiological studies have highlighted the J-shaped relationship between the risk of developing a given disease state and alcohol/wine consumption (de Lorimier, 2000). Moderate consumption provides optimal protection, with the risk of developing a given disease being higher in abstainers and excessive alcohol consumers.

A strategy to possibly enhance the health benefits derived from wine consumption would involve increasing the levels of the bioactive compounds present in wine. Increasing the ethanol concentration of wine would not be a viable option for obvious reasons. The alternative is to increase the level of phenolic compounds, resveratrol being a good candidate considering its well studied biological activities. Wine quality is dependent on a series of interlinked factors, originating with the production of good quality grapes and culminating in the bottling of the wine. Employing a range of techniques throughout the process to increase the resveratrol levels would most probably affect wine quality – whether positively or negatively would only be able to be determined in the finished product. Herein lies the risk of manipulating factors within the production process in order to increase resveratrol levels along conventional and accepted lines.

With the advent of recombinant DNA technology, it has become possible to target specific traits of an organism for modification. Genetic engineering of *Saccharomyces cerevisiae* to enhance existing or introduce novel traits has been extensively utilised. The use of yeast starter cultures possessing consistent and desirable oenological traits to complete the primary alcoholic fermentation during winemaking is common practice. Although the use of genetically modified organisms in the wine industry is not commonplace as of yet, in future it may be possible to combine specifically designed recombinant technologies and winemaking practices in order to enhance certain traits of a wine without an accompanying ripple effect that could negatively impact wine quality.

The overriding aim of our research is the development of a wine yeast strain capable of resveratrol production during grape must fermentation. It is proposed that red and white wines produced with such a resveratrol-producing yeast will contain elevated levels of resveratrol, and that added health benefits may be derived from their consumption. Initial work by Becker *et al*. (2003) established that yeast expressing genes encoding coenzyme A ligase (*4CL216*) from hybrid poplar and resveratrol synthase (*vst1*) from *Vitis vinifera*, which form part of the phenylpropanoid pathway in plants, was able to produce resveratrol.

Following this study, work by several research groups has focused on the production of secondary plant metabolites in microbial systems as an alternative to extraction from plants or chemical synthesis. Watts *et al.* (2006) engineered *Escherichia coli* to produce, depending on the precursor supplied, resveratrol and piceatannol. In a similar vein, Beekwilder *et al.* (2006) compared the production of resveratrol production in *E. coli* and *S. cerevisiae* and found it to be comparable.

1.2 SPECIFIC AIMS

In this study, we optimised the expression of resveratrol synthesis genes in *Saccharomyces cerevisiae* and investigated the dynamics of resveratrol production in this recombinant system. In order to achieve the latter, the initial development of an optimised method for the extraction of resveratrol from the intracellular fraction of yeast was required. In addition, a method for the extraction of resveratrol from the extracellular fraction of yeast was devised. The influence of yeast genetic background and precursor availability on resveratrol production was investigated.

Specific aims included:

- Construction of single copy yeast integration vectors containing expression cassettes comprising *vst1* and *4CL216* each under the control of the constitutive *PGK1* promoter and terminator;
- Transformation of both constructs into three laboratory strains of *S. cerevisiae* – FY23, CEN.PK42 and Σ272;
- Confirmation of the integration and expression of heterologous genes;
- Optimisation of the extraction of resveratrol from the intracellular and extracellular fractions of yeast cells;
- Elucidating yeast growth patterns in order to investigate the physiological influence, if any, of resveratrol on the yeast and to identify optimal sampling time points;
- Analysis and comparison of resveratrol production in different yeast genetic backgrounds over time;
- Investigation of factors influencing resveratrol yields – precursor feeding study; and
- Investigation into the physiological effect(s) of endogenously-produced resveratrol on yeast.

1.3 LITERATURE CITED

Becker JVW, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* **4**: 79-85.

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CHR, Bovy A. 2006. Production of resveratrol in recombinant microorganisms. *Appl Environ Microbiol* **72**: 5670-5672.

de Lorimier AA. 2000. Alcohol, wine, and health. *Am J Surg* **180**: 357-361.

Siemann EH, Creasy LL. 1992. Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic* **43**: 49-52.

Watts KT, Lee PC, Schmidt-Dannert C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol* **6**: 22-34.

LITERATURE REVIEW

2.1 INTRODUCTION

Resveratrol is a small phenolic compound produced via secondary metabolism in numerous plant families. Interest in this compound is mainly focused on firstly, its functions in grapevine-pathogen interactions and secondly, its effects on human health. The aspect linking these largely distinct focus areas is wine. As a grape-based beverage, wine contains resveratrol and its consumption forms an integral part of the human diet in many populations around the world.

Aspects of the production of resveratrol and its derivatives, together with their biological importance in grapevine will be discussed. From an oenological perspective, factors affecting the levels of resveratrol present in wine can broadly be divided along the line of those that affect the amount of resveratrol produced in the grape berries, and those affecting its extraction from the grapes during winemaking and its subsequent stability in the wine. Some of the main factors affecting plant production of resveratrol, excluding viticultural practices, and oenological practices that influence the final concentrations of resveratrol in wine will be examined.

The positive effects of resveratrol on human health have long been exploited as evidenced by the use of resveratrol-containing *Polygonum cuspidatum* roots in traditional oriental medicine. Scientific investigations have indicated that it mediates protection against several important pathologies: cardiovascular heart disease, cancer, viral infection and neurodegenerative processes. Since most results on the biological activities of resveratrol have been obtained from *in vitro* studies, the progress made in determining whether resveratrol reaches its cellular targets in an active form *in vivo* will be presented.

Recent research has indicated that resveratrol exhibits life extension properties in yeast, flies, worms and fish. Yeast serves as a model organism in ageing studies – the mechanisms of ageing and the role of resveratrol in the prevention thereof will be discussed.

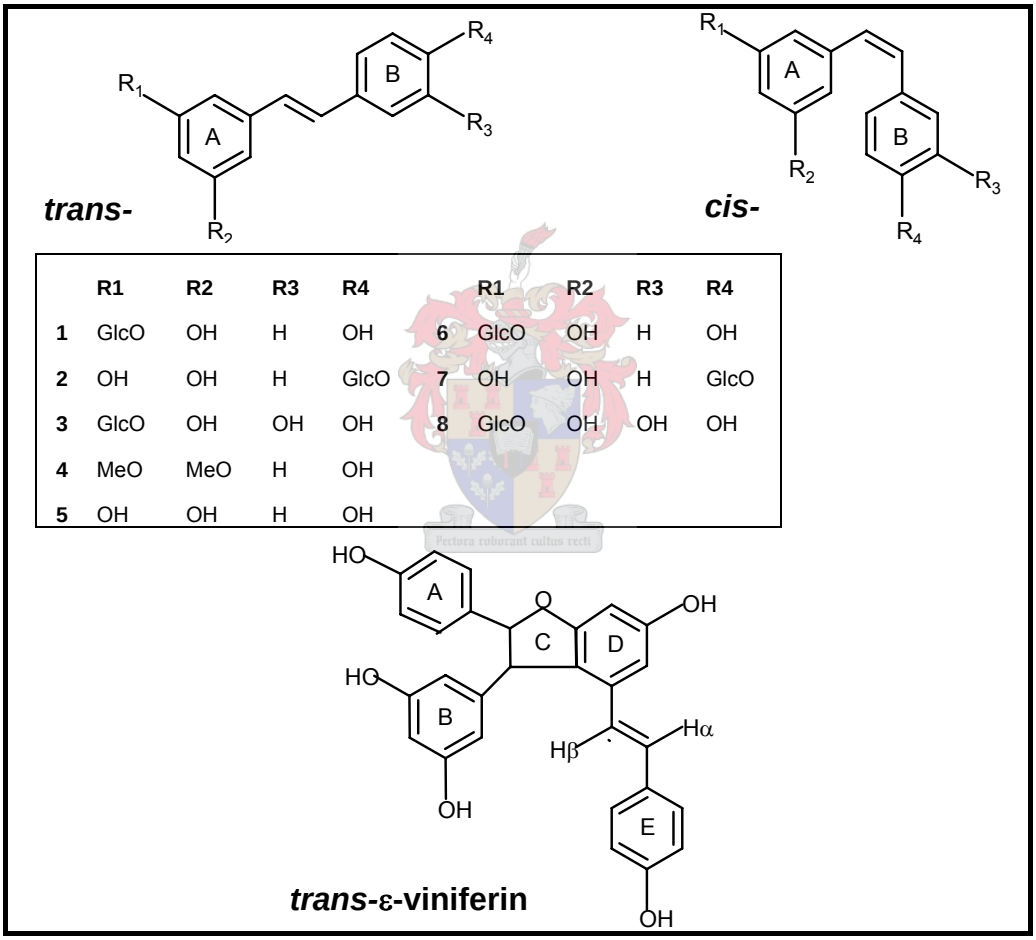
2.2 BIOSYNTHESIS OF RESVERATROL IN GRAPEVINE

2.2.1 STILBENE BIOSYNTHESIS IN *Vitis vinifera*

Phytoalexins are plant chemicals of low molecular weight which are inhibitory to microorganisms and accumulate in plants due to interactions of the plant with the microorganisms (Siemann and Creasy, 1992). Resveratrol is classified as a phytoalexin as its production in grape berries and leaves occurs in response to biotic (Dercks and

Creasy, 1989; Langcake and Pryce, 1976;) or abiotic stresses (Adrian *et al.*, 1996; Langcake and Pryce, 1977c). Common fungal pathogens include grey mould (*Botrytis cinerea*), powdery mildew (*Oidium tuckeri*) and downy mildew (*Plasmopara viticola*) (Okuda and Yokotsuka, 1996). Other factors that induce stilbene synthesis include ozone, UV light, heavy metal-containing fungicides and methyljasmonate (Adrian *et al.*, 1996; Chiron *et al.*, 2000; Dercks and Creasy, 1989; Douillet-Breuil *et al.*, 1999; Jeandet *et al.*, 2000; Krisa *et al.*, 1999; Liswidowati *et al.*, 1991; Schubert *et al.*, 1997; Wiese *et al.*, 1994; Zinser *et al.*, 2000).

Phytoalexins produced by plants in the *Vitaceae* family constitute a restricted group of compounds belonging to the stilbene family, the skeleton of which is based on the structure of *trans*-resveratrol (3,5,4'-trihydroxystilbene) (Langcake and Pryce, 1977a) (**Figure 2.1**).



	R1	R2	R3	R4		R1	R2	R3	R4
1	GlcO	OH	H	OH	6	GlcO	OH	H	OH
2	OH	OH	H	GlcO	7	OH	OH	H	GlcO
3	GlcO	OH	OH	OH	8	GlcO	OH	OH	OH
4	MeO	MeO	H	OH					
5	OH	OH	H	OH					

Figure 2.1. Chemical structures of stilbene phytoalexins in *Vitis vinifera*. **1** and **6**, *trans*- and *cis*-piceid; **2** and **7**, *trans*- and *cis*-resveratroloside; **3** and **8**, *trans*-and *cis*-astringin; **4**, *trans*-pterostilbene; **5** *trans*-resveratrol. Glc: glucosyl ($C_6H_{11}O_5$) (Jeandet *et al.*, 2002).

Simple stilbenes that have been identified include: *trans*-pterostilbene, a dimethylated resveratrol derivative (Langcake, 1981; Pezet and Pont, 1990); *trans*- and *cis*-piceid, 3-*O*-β-D-glucosides of resveratrol (Waffo-Teguo *et al.*, 1996; Waterhouse and Lamuela-Raventos, 1994); *trans*- and *cis*-astringin, 3-*O*-β-D-glucoside of 3'-hydroxy-resveratol; and

trans- and *cis*-resveratrol-oside, a 4'-*O*-β-D-glucoside of resveratrol (Waffo-Teguo *et al.*, 1998). Stress in grapevine also elicits the production of viniferins, which are oligomers of resveratrol. They include ε-viniferin, a cyclic dehydrodimer of two resveratrol units (Jeandet *et al.*, 1997; Langcake and Pryce, 1977a); α-viniferin, a cyclic dehydrotrimer (Pryce and Langcake, 1977); and the recently discovered δ-viniferin, also a dehydrodimer but consisting of a resveratrol unit and a resveratrol glucoside unit (Pezet *et al.*, 2003; Waffo-Teguo *et al.*, 2001b). The existence of a cyclic resveratrol tetramer (β-viniferin) and a high molecular weight oligomer (γ-viniferin) has been suggested but no direct evidence thereof provided (Langcake and Pryce, 1977c).

Resveratrol is synthesised from phenylalanine via the phenylalanine/polymalonate pathway (Langcake and Pryce, 1977a). In the final step, the stepwise condensation of three molecules of malonyl-CoA to *p*-coumaroyl-CoA is catalysed by stilbene synthase (**Figure 2.2**).

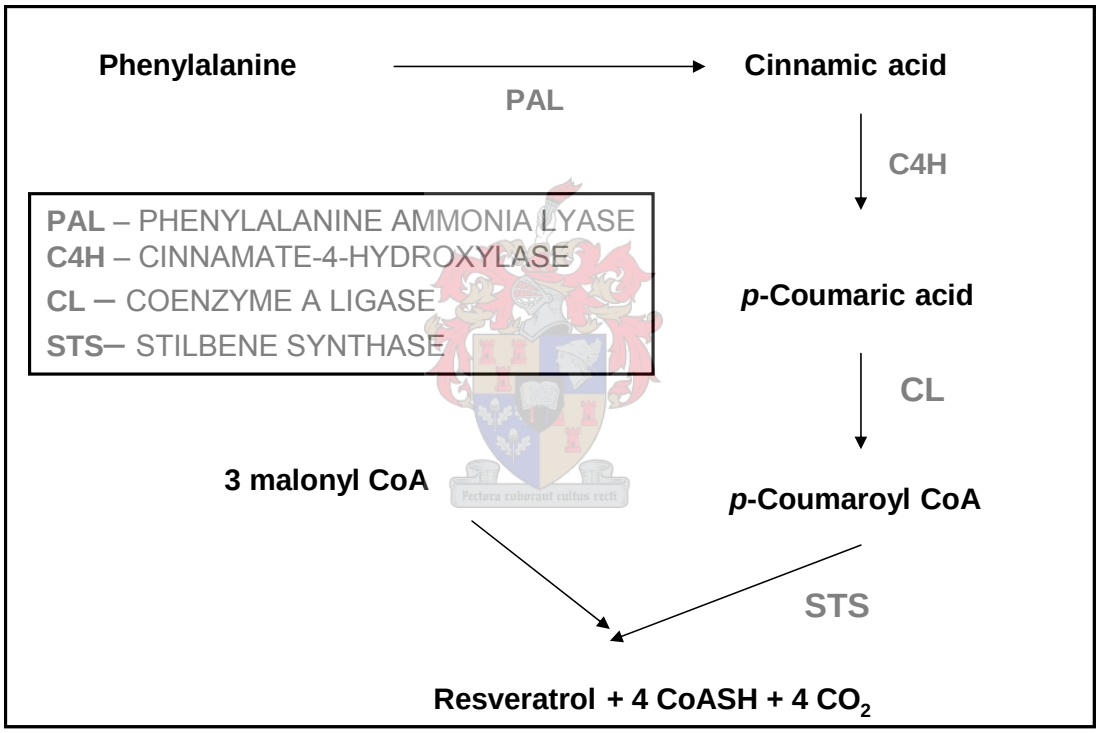


Figure 2.2. Biosynthesis of resveratrol from phenylalanine via the phenylpropanoid pathway (Becker *et al.*, 2003).

Stilbene synthase is encoded by a 15-20 member multigene family of which seven resveratrol-forming genes have been characterised in *Vitis vinifera.* Stilbene synthase was found to be constitutively expressed in germinating grapevine seeds (Sparvoli *et al.*, 1994). However, Borie *et al*. (2004) recently reported that stilbene synthase mRNAs were not detected in uninduced leaves. Constitutive expression of stilbene synthase thus appears to be limited to germinating seeds, as the expression in leaves and cell suspensions has been reported by several authors to occur in two waves in *V. vinifera* (Liswidowati *et al.*, 1991; Wiese *et al.*, 1994; Zinser *et al.*, 2000). Wiese *et al*. (1994) proposed that the two

peaks of mRNA accumulation, following fungal induction of grapevine cell suspension cultures, correspond to differential expression of at least two types of stilbene synthase genes – those expressed early (within 3-5 hours) but with a rapid degradation of mRNAs and those expressed later (11-16 hours), being slowly activated and providing a more stable mRNA. A similar biphasic profile was reported for resveratrol synthesis (Douillet-Breuil *et al.*, 1999) which, in accordance with findings by Borie *et al.* (2004), confirmed the correlation between gene expression and resveratrol production.

Resveratrol is produced at the abaxial surface of leaves, in the skin of grape berries (Jeandet *et al.*, 1991) and, to a lesser extent, in the seeds (Adrian *et al.*, 2000; Ector *et al.*, 1996; Jeandet *et al.*, 1991; Langcake and Pryce, 1976). Resveratrol production in grape berry skins was shown to remain stable prior to veraison, after which synthesis decreased during ripening. In ripe berries levels of resveratrol decreased to 10% of the highest value measured during early development (Jeandet *et al.*, 1991).

2.2.2 RESVERATROL DERIVATIVES AND THEIR SIGNIFICANCE IN GRAPEVINE

Resveratrol and its derivatives are mainly involved in protecting the plant against pathogen attack and constitute an array of compounds with differing degrees of substitution, polymerisation and toxicities. The formation of viniferins results from the oxidative dimerisation of resveratrol units, mediated by peroxidase enzymes (Calderon *et al.*, 1992; Langcake and Pryce, 1977b; Ros Barcelo *et al.*, 2003). A laccase-like stilbene oxidase from *B. cinerea,* an important grapevine pathogen, has also been shown to mediate oxidative dimerisation of resveratrol (Breuil *et al.*, 1998; Pezet *et al.*, 1991). In grapevine, resveratrol oxidation is controlled by three peroxidase isoenzymes – A_1 and B_3 located in the cell wall and cell wall-free-spaces; and B_5 located at the vacuolar level (Calderon *et al.*, 1992).

Pterostilbene, a dimethylated resveratrol derivative, has been characterised but the biosynthetic pathway has not been clarified (Jeandet *et al.*, 2002). The enzyme (or enzymes) responsible for the formation of piceid has not yet been identified, although findings indicate that it is similar to other phenolic glucosyltransferases, but probably a distinct enzyme (Krasnow and Murphy, 2004).

All stilbenes are not equally toxic to pathogens. As an antifungal compound, pterostilbene is five times more potent than resveratrol and is reported to be the most toxic stilbene (Adrian *et al.*, 1997; Langcake, 1981; Pezet and Pont, 1990). ε-Viniferin has activity similar to pterostilbene on germinating *B. cinerea* conidia (Langcake, 1981). Similarly, δ-viniferin was shown to have equal toxicity to pterostilbene against zoospores of *P. viticola* (Pezet *et al.*, 2004a). Pezet *et al.* (2004b) reported that piceid, even at elevated concentrations, did not show any toxic activity against *P. viticola* zoospores.

The pattern of accumulation of resveratrol and its derivatives appears to be indicative of a plant's resistance or susceptibility to pathogen attack. Resistant cultivars were shown

to accumulate high concentrations of viniferins and, simultaneously, synthesise large amounts of resveratrol which could serve as a pool for viniferin synthesis. In susceptible cultivars, large amounts of resveratrol were synthesised early following infection but were rapidly glycosylated to form piceid, a non-toxic compound. Levels of viniferins were accordingly low in susceptible cultivars (Pezet *et al.*, 2004b).

The role of resveratrol glycosylation *in planta* remains unknown. Most polyphenols are not substituted at their 'reducing' hydroxyl group (4'-hydroxy group in the case of resveratrol) thus, they retain their antioxidant capacity yet may still be exposed to auto- and pathogenic-oxidising enzymes (Arora *et al.*, 1998; Pannala *et al.*, 2001). Polyphenol oxidases are highly conserved throughout the three kingdoms (Mayer and Harel, 1979; Robb, 1984) and in plants are thought to be involved in the defence system (Mayer and Harel, 1979). Regev-Shoshani *et al*. (2003) showed that piceid is resistant to oxidation by tyrosinase, a polyphenol oxidase. They therefore, suggest that glycosylation of resveratrol has evolved in plants to protect them from deleterious oxidation by the plants' own polyphenol oxidases. Simultaneously, the beneficial antioxidant activities have been retained and, in agreement with Vickery and Vickery (1981), solubility has increased.

2.2.3 FACTORS AFFECTING RESVERATROL PRODUCTION IN GRAPEVINE FROM AN OENOLOGICAL PERSPECTIVE

2.2.3.1 Geographical Location

An early study by Sieman and Creasy (1992) indicated that geographical origin appeared to be a factor in determining the level of resveratrol in wine. They found that there was significantly more resveratrol in New York Chardonnays than in those from California. However, they could not account for differences due to growing practices and winemaking styles.

A more comprehensive study examined the resveratrol levels in over 300 wines, sampled within approximately one year, of various geographical origin (Goldberg *et al.*, 1995b). No obvious general pattern was discerned for resveratrol levels in wines of different origins, however when examining Cabernet Sauvignon wines it was apparent that climate played a role. Californian, Australian and South American Cabernet Sauvignon wines had lower *trans*-resveratrol concentrations than those from Bordeaux and Ontario. Fluctuations appeared to be temperature dependent, thus the cooler and more humid climate of Bordeaux and Ontario may have accounted for the differences. Other studies further support the finding that the concentration of *trans*-resveratrol is relatively low in wines produced in the warmer climate regions of the Mediterranean (Goldberg *et al.*, 1996; Sakkiadi *et al.*, 2001).

In different areas described as climactically similar (warm and dry), resveratrol concentrations still fluctuated. The differences were attributed to the intrinsic resveratrol-

synthesising capacity of the different cultivars employed in these areas. However, differences were still found within the same area where cultivar was not the determining factor (Goldberg *et al.*, 1995b).

In a study where Italian red wines of different origins were examined, differences in resveratrol contents of wine could not be correlated to geographical origin but were rather attributed to factors in the vineyards (which were not elaborated on) or ageing (Gambelli and Santaroni, 2004).

Findings thus indicate that geographical location is not a clear determinant of resveratrol levels in wine. In studies of this nature, it becomes difficult to rule out other factors that affect resveratrol levels in wine, thus conclusions drawn in different studies as to the effect of geographical origin are often conflicting. These complexities, therefore, do not allow for a clear or simple answer when trying to determine the role of geographical location in determining resveratrol levels in wine.

2.2.3.2 Fungal pressure in the vineyard

Levels of resveratrol in wine would be expected to be higher when fungal pressure in the vineyard is high, as fungal infection has been shown to be an elicitor of resveratrol synthesis (Siemann and Creasy, 1992). Jeandet *et al*. (1995b) found that when there was high or moderate *Botrytis* pressure in vineyards, the resveratrol content in wine was relatively low. The explanation for this seemingly paradoxical finding was that although resveratrol was produced, it may have been degraded by exocellular enzymes of *B. cinerea*, e.g. a laccase-like stilbene oxidase.

When the *Botrytis* pressure was low, resveratrol levels in wine were high. The authors hypothesise that during periods of low *Botrytis* pressure, fewer grapes bunches are directly attacked by the pathogen and those that do not appear infected, produce high levels of phytoalexins (Jeandet *et al.*, 1995a). In addition, it is thought that the pathogen has not developed fully to the stage of phytoalexin degrading enzyme production by the time the grapes are harvested (Jeandet *et al.*, 1993). Thus, higher amounts of resveratrol are available for extraction into wines. Resveratrol concentration in must and wine is therefore, a reflection of the balance between the production by the plant and degradation by fungal enzymes.

When *Botrytis* development is extremely limited, resveratrol levels in wine are low as a result of overall reduced induction of phytoalexin synthesis (Jeandet *et al.*, 1995b).

2.2.3.3 Ultraviolet light irradiation

UV irradiation is used as an *in vitro* tool for the elicitation of resveratrol synthesis and has been used in numerous studies as a means to study resveratrol production. Langcake and Pryce (1977c) were the first to report this phenomenon, and reported that the induction of resveratrol synthesis showed a maximum in the region 260–270 nm, which

would explain why sunlight does not act as an inducer. More recently, the applied use of postharvest UV-C irradiation has been investigated as a stilbene enrichment technique in table grapes and grape musts (Cantos *et al.*, 2002, 2003). Resveratrol content in wines made from UV-C irradiated grapes was twice as high as control wine. The susceptibility of grape varieties to UV-C induction differs and authors proposed that superior stilbene-enrichment of wines could be achieved with more susceptible varieties (Cantos *et al.*, 2003). Importantly, the treatment did not affect general oenological wine parameters, wine aroma or taste.

2.2.3.4 Grape variety

Creasy and Coffee (1988) found that the resveratrol production potential of grape berries varied greatly, with high and low potentials for both red and white berries. This intrinsic ability of various cultivars to produce resveratrol is thought to be genetically controlled (Lamuela-Raventos *et al.*, 1995). In agreement with the findings of Creasy and Coffee (1988), Okuda and Yokotsuka (1996) did not detect significant differences in resveratrol content of skins between white skinned and red/pink skinned varieties. As per example, some of their results are shown in **Table 2.1**.

Table 2.1. Resveratrol content of grape berry skins from different cultivars (Okuda and Yokotsuka, 1996).

Variety	Skin Colour	Resveratrol Content (µg/g skin fresh weight)
Chardonnay	White	4.51 ± 0.13
Müller-Thurgau	White	14.13 ± 0.13
Riesling	White	2.97 ± 0.04
Sauvignon blanc	White	5.13 ± 0.02
Sylvaner	White	0.74 ± 0.03
Cabernet Sauvignon	Red	3.48 ± 0.02
Delaware	Red	9.50 ± 1.20
Merlot	Red	5.78 ± 0.01
Urbana	Red	0.84 ± 0.01
Zweigeltrebe	Red	8.69 ± 0.11

The single cultivar that seems to have consistently high levels of *trans*-resveratrol in wines, irrespective of the country or region of origin, is Pinot noir (Goldberg *et al.*, 1995b; Lamuela-Raventos and Waterhouse, 1993). The high levels may be due to the early harvesting of this particularly susceptible cultivar to wet weather conditions prior to harvesting. Subsequently, levels of resveratrol would not yet have decreased as a result of ripening. Also the characteristically thin skin of this cultivar may render it especially prone to fungal infection and hence induction of resveratrol synthesis (Goldberg *et al.*,

1995b). The possibility also exists that resveratrol extraction is facilitated by the thinner skins – if this is actually the case has yet to be determined.

2.3 FACTORS INFLUENCING RESVERATROL LEVELS IN WINE

2.3.1 EVOLUTION OF RESVERATROL AND ITS DERIVATIVES DURING FERMENTATION

Studies have shown that resveratrol production in grape berries is located at the level of the skin with minimal amounts produced in the berry flesh (Creasy and Coffee, 1988; Jeandet *et al*., 1991). Numerous factors will influence the final concentration of resveratrol in wine but skin contact period appears to be most important as evidenced by generally low levels in white wines and higher levels in red wines, despite similar amounts of resveratrol available for extraction from the skins (Okuda and Yokotsuka, 1996). During red wine vinification skin contact is extended as fermentation occurs on the skins. However, during white wine vinification skin contact is minimal as skins are removed prior to fermentation (Siemann and Creasy, 1992).

A general pattern emerges for the extraction of resveratrol and piceid during fermentation (**Figure 2.3**). At the start of fermentation, the levels of resveratrol glucosides and aglycones are low, although glucosides predominate. As fermentation progresses and ethanol is produced, the increased solubility of all forms of resveratrol results in their concentrations increasing (Mattivi *et al.*, 1995).

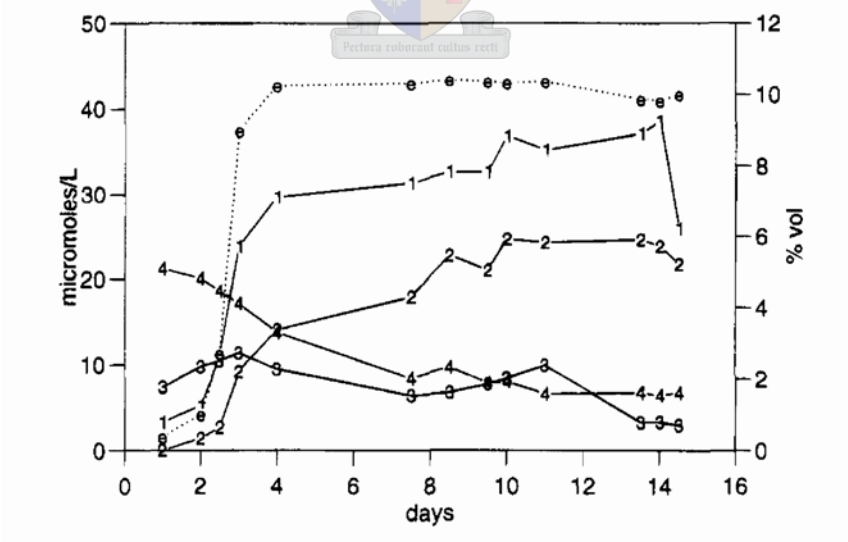


Figure 2.3. Evolution of *trans*-resveratrol (1), *cis*-resveratrol (2), *trans*-resveratrol glucoside (3), *cis*-resveratrol glucoside (4) and ethanol (e) in free run juice during a conventional red wine vinification (Mattivi *et al.*, 1995).

Over time, the levels of glucosides begin to decrease while aglycone levels increase. The activity of β-glucosidases is proposed to be, at least in part, responsible for the

decrease in glucoside concentrations (Delcroix *et al.*, 1994). After fermentation and bottling, resveratrol levels remain relatively stable in wine over time (Goldberg *et al.*, 1995b; Jeandet *et al.*, 1995b). Okuda and Yokotsuka (1996) showed that the concentration of resveratrol in white wines after vinification was between 3 and 6% of the maximum extractable amount in the skins. In the case of red wines, between 7 and 36% of resveratrol was extracted into the wine.

Despite many reports on the production of viniferins in grapevine, relatively little attention has been paid to the viniferin content in wine. Recently ε-viniferin (Landrault *et al.*, 2002) and δ-viniferin (Vitrac *et al.*, 2005) were identified in wine with their levels making an important contribution to the total stilbene contents of the wines analysed.

2.3.2 YEAST STRAIN

When testing the fate of supplemented resveratrol in a laboratory liquid culture, Vacca *et al.* (1997) found yeast to cause a decrease in resveratrol concentration. A high alcohol producing strain of S*accharomyces cerevisiae* caused a 32% decrease in the level of resveratrol, while a low alcohol producing strain of *Metschnikowia pulcherrima* caused a reduction of 20%. The authors speculated that the decrease was caused either by adsorption to the yeast cell walls or uptake and metabolism by the yeasts, although the specific pathways involved were not elucidated.

Franco *et al*. (2002) studied the effect of yeast strain (*S. cerevisiae*) on the evolution of resveratrol and its glucosides in must and wine. They found that irrespective of the yeast strain used to conduct the fermentation, the levels of *cis*- and *trans*-piceid decreased as fermentation proceeded. There was not a proportional increase in the aglycone levels that accompanied the decreased glucoside levels. After two months a decrease in all forms was noted, which is contrary to the findings of Jeandet *et al.* (1995b) and Goldberg *et al.* (1995a). It is not clear whether the wine was racked and bottled prior to storage, which may account for the different results of Franco *et al*. (2002). Moreover, there was a more apparent reduction in free forms of resveratrol than glucosides. With respect to the aglycone levels, differences in yeast activity were more pronounced in the early stages of fermentation. Some strains caused an increase in either one or both of the free forms. Other strains caused a decrease in free forms of resveratrol, some affecting both isomers while others only had an effect on *trans*-resveratrol levels. The authors did not suggest any possible reasons for their findings.

The influence of wine yeasts with differing phenolic extraction capabilities on resveratrol concentration were investigated (Clare *et al.*, 2005). Comparable to the findings of Franco *et al*. (2002), the evolution of mainly the free resveratrol isomers was affected by the different yeast strains. Clare *et al*. (2005) also showed a strong positive correlation between total resveratrol concentrations and total phenolics in wine, both of which were yeast strain dependent.

2.3.3 VINIFICATION TECHNIQUES

When considering the factors that influence the final resveratrol concentration in wine, the selection of specific vinification techniques may be where the winemaker can play the most active role in producing wines with elevated resveratrol levels. However, a great deal of skill and experience would be required in order to select which techniques contribute positively both to resveratrol levels and to the sensory qualities of the wine.

Prefermentative oxygenation of grape must has been shown to decrease particularly *trans*-resveratrol levels by up to 50%. On the other hand, protecting grapes from oxidation by sparging with ascorbic acid and sulphur dioxide, may result in significantly higher levels of resveratrol in wines (Castellari *et al.*, 1998).

Prefermentative pomace contact methods greatly impact the levels of resveratrol in finished wines (Clare *et al.*, 2004). Nonetheless, the amount of resveratrol extracted from the grape skins is dependent on grape variety (Okuda and Yokotsuka, 1996). During cold maceration or cold soaking, must containing the skins and seeds is soaked in a cool environment (<20°C) for one to two days prior to alcoholic fermentation. This is proposed to achieve an aqueous extraction without the effects of ethanol on grape cells. Thermovinification involves heating the must for a short period of time after crushing to enhance extraction from the skins. The must is then cooled and pressed and skins and seeds are removed. Fermentation is initiated by inoculation with yeast. During carbonic maceration whole, intact bunches of grapes are kept in a carbon dioxide atmosphere and allowed to respire and partially ferment until the alcohol concentration reaches 1 to 1.5% (v/v). After eight to ten days, berry fermentation ceases as the glycolytic enzymes that conduct the fermentation lose activity. Bunches are then pressed and the run-off is inoculated and fermented without the skins. Results indicate that, in comparison to a classical red wine vinification, wines produced by thermovinification increased total resveratrol concentration by 266%. Cold soaking increased total resveratrol levels by 27% while no resveratrol was detected in wines that underwent carbonic maceration (Clare *et al*. 2004).

2.3.4 MACERATION TIME AND FINING AGENTS

A direct correlation between extended maceration time and resveratrol levels would be expected. In contrast to expectations, Threlfall and Morris (1996) found that extending the skin contact period by one week, after fermentation had reached dryness, did not significantly affect resveratrol levels. Gambuti *et al*. (2004) found that *trans*-resveratrol concentrations decreased when maceration time was extended by 10 days. Precipitation, adsorption on yeast lees or marc, and isomerisation to *cis*-resveratrol were proposed to be the causes.

In turn, excessive maceration may lead to the extraction of astringent and bitter phenolic compounds that could impact negatively on wine quality. Fining agents are often added to wine to remove these compounds (Doner *et al.*, 1993; McMurrough *et al.*, 1984), with differing effects on resveratrol levels. Vrhovsek *et al*. (1997) showed that fining wine with gelatine did not affect the concentration of free or glucosidic forms of resveratrol. Bentonite and diatomaceous earth also have no major effect on resveratrol levels (Goldberg *et al.*, 1997; Soleas *et al.*, 1995). Polyvinylpolypyrrolidone (PVPP) was shown to decrease the concentration of all resveratrol forms in wine – *cis*-resveratrol by up to 90% (Vrhovsek *et al.*, 1997; Threlfall and Morris, 1996). Carbon fining (activated charcoal) and filtering also did not affect resveratrol levels in wine (Threlfall and Morris, 1996). On the contrary, Castellari *et al*. (1998) found charcoal and PVPP to virtually eliminate both isomers of free resveratrol from wine.

2.3.5 ENZYME TREATMENTS

Pectolytic enzyme preparations are used in winemaking to increase juice yield, facilitate colour (phenolic compounds) extraction and stability, and facilitate clarification (Felix and Villettaz, 1983; Lanzarini and Pifferi, 1989; Voragen and van den Broek, 1991). They act by breaking down grape skin cell walls (Sacchi *et al.*, 2005). There is a bilateral effect of these enzyme preparations on the resveratrol concentration in wine. These commercial enzyme preparations often contain numerous impurities of which extraneous β-glucosidases form a part. Thus, the resveratrol levels may be affected by the conversion of resveratrol glucosides to aglycones, plus the enzymes may facilitate the extraction from the skins. Some researchers measure only the *trans*-resveratrol levels in the wine and it is not clear whether the increased levels are due to improved extraction of resveratrol as a result of the added pectolytic enzymes or whether it is due to the conversion of the glucosides.

Wightman *et al.* (1997) showed that in Pinot noir wines, some enzyme preparations caused a significant increase in final resveratrol concentrations while others did not affect the levels. Also, the dosage of enzyme also significantly affected resveratrol levels – a four fold increase in the enzyme dosage caused *trans*-resveratrol levels to double. However, in Cabernet Sauvignon wines enzyme treatment did not increase final resveratrol concentration significantly.

Clare *et al*. (2002) found that throughout the fermentation of must treated with pectolytic enzymes, both *cis*- and *trans*-resveratrol levels were lower than in untreated must. However, after pressing, enzyme treated wine contained resveratrol levels 33% higher than the control wine. It thus appears that levels increased as a result of facilitated extraction due to the pectolytic enzyme treatment.

2.4 RESVERATROL FROM THE HUMAN PERSPECTIVE

Numerous positive effects on human health have been associated with regular, moderate consumption of wine, especially red wine. The ethanol present in wine is partially responsible for the protective effects of red wine, at least against the development of cardiovascular diseases, but numerous biological activities (also pertaining to other diseases) of the phenolic constituents have also been reported (Fremont, 2000). The report by Siemann and Creasy (1992) on the presence of resveratrol in wine and the possibility of it being the biologically active component of red wine focused attention on this compound. The discrepancy between the concentrations of resveratrol required for *in vitro* activity and the levels reported in wines at that time cast doubt over the notion of resveratrol being the active ingredient in wine. However, the identification of relatively high levels of resveratrol derivatives in wines since then and further studies revealing that these derivatives have similar biological activities to resveratrol, helped to restore the credibility of the initial report by Siemann and Creasy (1992). The following sections compare the biological activities of the different forms of resveratrol and describe the absorption and metabolism of resveratrol in the human body.

2.4.1 COMPARISON OF THE BIOLOGICAL ACTIVITY OF RESVERATROL AND PICEID ISOMERS

Most studies investigating the pharmacological activity of resveratrol have focused on the *trans*-isomer, owing mainly to its commercial availability. Few studies have focused on *cis*-resveratrol or the glucosides. An array of biological activities have been reported for *trans*-resveratrol and the main ones include inhibition of lipid peroxidation – both low density lipoprotein (LDL) and membrane lipids; chelation of copper; free-radical scavenging; alteration of eicosanoid synthesis; inhibition of platelet aggregation; anti-inflammatory activity; vasorelaxing activity; modulation of lipid metabolism; anticancer activity; and oestrogenic activity (Fremont, 2000).

The isomers of free resveratrol have different spatial conformations. *trans*-Resveratrol has a planar conformation whereas *cis*-resveratrol is more 3-dimensional. Orallo (2006) reported that this does not markedly modify the interaction with potential cellular targets. He therefore, concluded that the inhibitory effects of the two isomers are qualitatively similar. The antioxidant capacity of resveratrol can be quantified using different assay techniques, and for each technique there are conflicting reports for the efficacy of the respective isomers (Orallo, 2006). Thus, what can be deduced is that both isomers do exhibit biological activity, although quantitatively they vary.

Piceid has also been shown to possess anticancer activity (Waffo-Teguo *et al.*, 2001a) and antioxidant activity (Waffo-Teguo *et al.*, 1998). Glycosylation of *trans*-stilbenes reduces their antioxidant activity, more so than in the *cis*-isomers. In addition, the position

of glycosylation is important. Addition of a glucosyl moiety to the 4'-hydroxyl group in the B ring (**Figure 2.1**) dramatically decreases antioxidant activity. Glycosylation of resveratrol in the 3-position in the A ring produces piceid – it reduces antioxidant activity by approximately half when compared to the aglycone (Waffo-Teguo *et al.*, 1998).

2.4.2 THE FATE OF ORALLY ADMINISTERED RESVERATROL

2.4.2.1 Absorption in the gastrointestinal tract

The health promoting effects attributed to resveratrol are subject to the absorption, metabolism and tissue distribution of orally administrated resveratrol (Yu *et al.*, 2002). Wine is believed to be a superior source of bioavailable polyphenolic compounds, as it contains phenolic compounds in less polymerised and conjugated states than in fruits and vegetables. This may be attributed to the breakdown of these aggregates during alcoholic fermentation. It is also thought that ethanol in wine contributes to the bioavailability of wine phenolics by maintaining them in solution, even in the intestines (Goldberg, 1995).

As a basis for absorption and bioavailability studies, it is necessary to ascertain whether the compound of interest indeed reaches the site of absorption and whether it is in its active form. Results from a study where wines were subjected to dissolution testing employing gastric and intestinal fluids, showed that both *trans*- and *cis*-isomers of resveratrol and piceid were resistant to gastrointestinal treatment (Martinez-Ortega *et al.*, 2001).

The Caco-2 cell line derived from human colon adenocarcinoma often serves as a model to investigate intestinal absorption in humans. These cells spontaneously differentiate into polarised cell monolayers with many enterocyte-like properties of transporting epithelia (Artursson and Karlsson, 1991). Kaldas *et al*. (2003) showed that *trans*-resveratrol is efficiently absorbed across intestinal Caco-2 cells, and that absorption increases with increasing concentrations of resveratrol. In a bid to elucidate the mechanisms involved in the intestinal uptake of *trans*-resveratrol and *trans*-piceid, Henry *et al*. (2005) found that the uptake of *trans*-resveratrol was faster and greater than for *trans*-piceid. ATP depletion did not significantly affect the uptake of *trans*-resveratrol, but *trans*-piceid uptake was reduced by up to 30%. These results suggest that *trans*-piceid is actively transported into the cells via a carrier protein system. Further investigations showed that the sodium-dependent glucose co-transporter (SGLT1) is involved in the transport of *trans*-piceid. Furthermore, results did not indicate that this transporter was involved in the uptake of the resveratrol aglycone. The authors therefore suggested that *trans*-resveratrol is absorbed across the apical membrane via passive diffusion.

There is little clarity in the matter of piceid absorption in the small intestine. Neither the acidity of the stomach nor the enzymes secreted by the stomach and the pancreas are able to hydrolyse β-glucosides (Dupont *et al.*, 1999). Henry *et al*. (2005) postulated that

following its uptake by SGLT1 in the apical membrane of enterocytes, piceid is acted upon by a cytosolic β-glucosidase (CBG) yielding free resveratrol and glucose. The proposed role of CBG *in vivo* is the detoxification of xenobiotics by hydrolysing β-glucoside moieties, thus providing a site for conjugation which would facilitate excretion of compounds via the bile and urine (Gopalan *et al.*, 1992; LaMarco and Glew, 1986). As CBG is an enzyme with broad substrate specificity, it seems plausible that it may be involved in the deglycosylation of piceid (Daniels *et al.*, 1981; Mellor and Layne, 1971).

Results from a study by Day *et al*. (1998) indicated that cell free extracts of human hepatocytes do not mediate the hydrolysis of quercetin-3-glucoside, and that the extracts from cells of the small intestine only resulted in the hydrolysis of small amounts of the compound in comparison to quercetin-4'-glucoside. These authors suggested that, in addition to CBG, there appeared to be another enzyme involved in the hydrolysis of quercetin-3-glucosides. Piceid (resveratrol-3-*O*-β-glucoside) exhibits structural similarity to quercetin-3-glucoside and phlorizin. Based on conclusions drawn by Day *et al*. (2000), the hydrolysis of piceid by membrane-bound lactase phlorizin hydrolase (LPH) on the luminal surface of the intestinal epithelium prior to absorption may be plausible. Following deglycosylation of piceid, resveratrol could diffuse passively into the enterocytes.

A factor limiting the bioavailability of compounds following absorption is their efflux back across the apical membrane of enterocytes. Henry *et al.* (2005) showed that following their absorption into Caco-2 cells, *trans*-resveratrol and *trans*-piceid were rapidly excreted from the cells. Results suggest that multidrug resistance-associated protein 2 (MRP2) is involved in the efflux of the stilbenes across the apical side of the Caco-2 cells, which would result in it being transported back into the intestinal lumen in humans. However, the authors do not rule out the involvement of MRP3, present in the basolateral membrane, in the efflux of absorbed stilbenes which may result in their uptake into the bloodstream.

2.4.2.2 Conjugation of resveratrol

One of the primary defence systems that the human body has developed in order to eliminate potentially harmful substances involves detoxification enzymes - either Phase I or Phase II enzymes. Most xenobiotics are hydrophobic in nature and require conversion to more hydrophilic forms to facilitate elimination from the body. Phase I enzymes are responsible for activation of xenobiotics after which endogenous detoxification (Phase II) enzymes mediate the elimination of activated xenobiotics by conjugation of reactive intermediates or reduction of oxidative intermediates. These enzymes are at highest concentrations in the liver, also occur in barrier epithelia and can be induced to very high levels by dietary inducers.

The enzymes involved in resveratrol metabolism, as reflected by the literature, are UDP-glucuronosyltransferases and sulphotransferases. The former enzyme catalyses the formation of glucuronides, which maximises biliary secretion and urinary excretion.

Glucuronidation has the highest capacity of all the detoxification reactions and represents a major mechanism of detoxification for many xenobiotics and metabolites of endogenous origin. Sulphotransferases provide an alternative mechanism to glucuronidation to enhance excretion of hydroxyl-containing compounds. Sulphotranferases have a lower capacity for conjugation than glucuronosyltransferases, but have higher affinity for xenobiotics. Thus at low concentrations of xenobiotics, sulphotransferases play a greater proportionate role and sulphation is often the predominant route of metabolism (Jones and Delong, 2000). In humans, sulphation and glucuronidation of resveratrol by the human duodenal mucosa and liver have been show to occur (Aumont *et al.*, 2001; Brill *et al.*, 2006; De Santi *et al.*, 2000).

Assays have demonstrated the absorption of *trans*-resveratrol after oral administration in humans. The predominant forms detected were glucuronide and sulphate conjugates with free resveratrol accounting for less than 10% of peak serum concentrations (Goldberg *et al.*, 2003; Soleas *et al.*, 2001a; 2001b).

Glucuronides of phenolic compounds have generally been assumed to be rapidly excreted *in vivo* and to be pharmacologically inactive. However, studies have demonstrated the pharmacological activity of certain drug glucuronides (Kroemer and Klotz, 1992; Sperker *et al.*, 1997). The question arises as to the nature of the biologically active form of resveratrol as most of the *in vitro* activity of resveratrol has been attributed mainly to the unconjugated form. β-glucuronidases have been isolated from a variety of organs (Sperker *et al.*, 1997) and it is conceivable that resveratrol glucuronide might be cleaved back to the aglycone form *in vivo* by the aforementioned enzymes, thus liberating the active form of the molecule (Kuhnle *et al.*, 2000).

2.4.2.3 Circulation and tissue accumulation of resveratrol in the body

Following the absorption of orally ingested compounds in the intestines, they proceed in the bloodstream, initially to the liver after which they circulate in the systemic blood system to various body tissues. Due to its low water solubility (Belguendouz *et al.*, 1997), resveratrol must be bound to proteins and/or conjugated to remain at a high concentration in serum. Jannin *et al.* (2004) demonstrated the interaction of resveratrol with albumin, which they propose to be one of the plasmatic carriers transporting resveratrol in the blood. In addition, they suggest that the binding of resveratrol to albumin could serve as a reservoir of resveratrol *in vivo,* and may play a crucial role in the distribution and bioavailability of circulating resveratrol.

Lancon *et al.* (2004) showed that the uptake of free resveratrol into hepatocytes involved passive diffusion and a carrier-mediated transport process. They proposed that in physiological conditions, the active transport process would dominate. Jannin *et al.* (2004) hypothesised that cellular uptake of resveratrol may involve the retention of resveratrol-albumin complexes by albumin membrane receptors, and that resveratrol

would then be delivered to the cell membrane in a similar fashion to fatty acids (**Figure 2.4**).

Vitrac *et al*. (2003) recently demonstrated the distribution of resveratrol in various organs, specifically the liver and kidney, and to a lesser extent in brain, heart, lung and testis following oral administration.

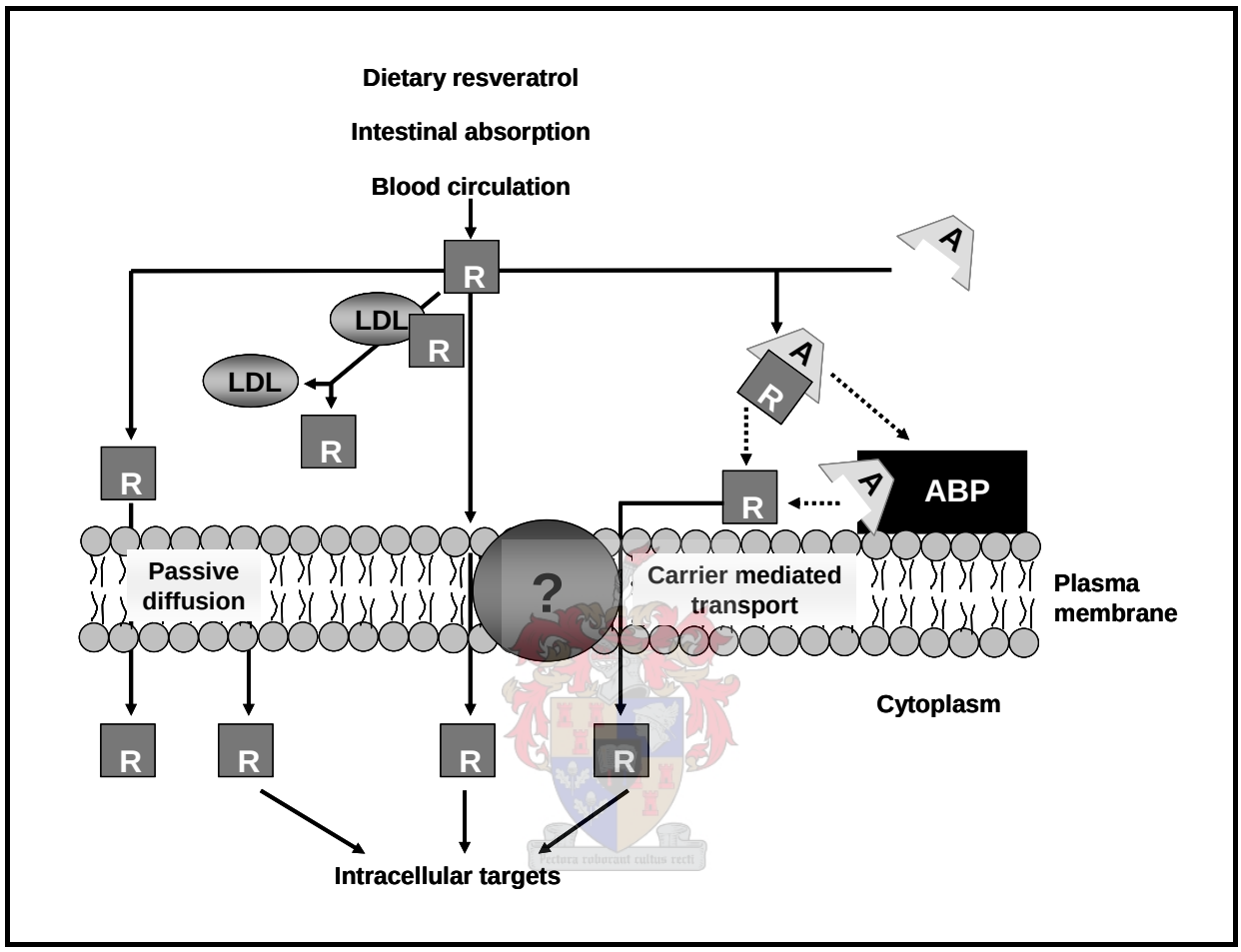


Figure 2.4. The proposed transport of resveratrol aglycone to cellular targets. R: resveratrol; A: albumin; ABP: albumin-binding protein (Jannin *et al.*, 2004).

The complexities involved in human studies do not facilitate the challenging task of elucidating the fate of resveratrol *in vivo*. Initially, most of the evidence obtained pointed towards its rapid excretion but when examined from different angles using different experimental techniques, more reports are indicating that resveratrol may in fact reach various parts of the body in a form where it may exert its effects. As is the case with most studies, more research may support or disprove the mechanisms described in this section. Nonetheless, it will provide impetus towards unravelling the claims of the health promoting effects of wine, the main source of resveratrol.

2.5 YEAST AGEING RESEARCH

2.5.1 AGEING HYPOTHESES

It has long been recognised that calorie restriction (CR) is able to extend the lifespan of organisms. Numerous theories have been proposed over the past 70 years to account for the life extending property of CR. A review by Sinclair (2005) highlights the shift in view from the initial proposal that ageing was caused by 'death genes' that directed the process of dying, to the more recent view of 'longevity genes' that have evolved to protect an organism during times of adversity. The activation of longevity genes culminates in cell defences that prevent cellular damage and lead to increased health and lifespan. The most recently proposed hypothesis, that accounts for the diverse array of findings from CR and lifespan-extension studies in numerous species, is termed the Hormesis Hypothesis of Calorie Restriction (Anderson *et al.*, 2003; Lithgow, 2001; Masoro, 2000; Masoro and Austad, 1996; Mattson *et al.*, 2002; Rattan, 2004, 1998; Turturro *et al.*, 1998; Turturro *et al.*, 2000). The hypothesis proposes that CR translates into a low-intensity biological stress on the organism, and that this triggers a defence response that helps protect it against the causes of ageing (Sinclair, 2005). An expansion of the hypothesis includes the idea that organisms can detect chemical stress signals from other species experiencing the stress of CR, either in their food or environment. Subsequently, their own defence pathways are activated in preparation for adverse conditions. The idea is known as the Xenohormesis Hypothesis (Howitz *et al.*, 2003; Lamming *et al.*, 2004).

2.5.2 TECHNIQUES TO STUDY YEAST AGEING

Saccharomyces cerevisiae is an accepted model organism for the study of ageing (Bitterman *et al.*, 2003; Guarente and Kenyon, 2000; Jazwinski, 2002; Vaupel *et al.*, 1998). Yeast lifespan can be examined from either the replicative or chronological angles. Replicative lifespan (RLS) refers to the number of daughter cells that a single mother cell can produce (Mortimer and Johnston, 1959) and is thus a model for dividing cells (Guarente and Kenyon, 2000). On the other hand, chronological lifespan (CLS) describes how long cells can remain viable in stationary phase (Fabrizio and Longo, 2003; MacLean *et al.*, 2001). Chronological ageing is based on non-dividing cells and thus serves as a model for post mitotic ageing, such as the ageing of neurons (MacLean *et al.*, 2001). The association between RLS and CLS in yeast remains unclear. Certain genetic alterations that increase RLS do not have the same effect on CLS (Fabrizio *et al.*, 2005) and some mutations have been shown to have opposite effects in the two lifespan assays (Fabrizio *et al.*, 2004; Harris *et al.*, 2003; Harris *et al.*, 2001). However, evidence exists that connects replicative and chronological ageing. Ashrafi *et al*. (1999) found that chronologically aged yeast cells displayed a reduced RLS. Yeast incubated for long

periods in stationary phase have a shortened replication potential when they enter exponential growth after being placed in fresh medium. Also certain gene deletions or mutations that decrease the activity of the protein kinase A pathway extend both RLS and CLS (Fabrizio *et al.*, 2001; Lin *et al.*, 2000; Longo *et al.*, 1997).

2.5.3 MECHANISMS OF CALORIE RESTRICTION AND YEAST AGEING

Saccharomyces cerevisiae has an asymmetrical budding pattern which can be exploited in order to determine RLS. The smaller bud is removed by micromanipulation and so the total number of daughter cells a single mother can produce may be enumerated. In most studies, the findings are related to RLS in order to determine the effect of the interventions, and develop a model for ageing in dividing yeasts.

2.5.3.1 Sir2p-dependent model

Yeast ageing has been shown to occur as a result of events in the nucleolus (Guarente, 1997). Ribosomal DNA (rDNA) and the components for ribosomal assembly are found in the nucleolus (Shaw and Jordan, 1995; Warner, 1990). The rDNA locus is arranged as 100 to 200 direct repeats of a 9.1 kb fragment, and approximately half of the repeats are transcriptionally active at one time (Dammann *et al.*, 1993). The remainder of the repeats are silenced by the silent information regulator protein (Sir2p) (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997). Silencing involves the NAD-dependent deacetylation of certain lysine residues in the N-termini of histones H3 and H4 (Grunstein, 1998). Together with Sir3p and Sir4p, Sir2p mediates the silencing of chromatin at telomeres and the mating-type loci (Gottschling *et al.*, 1990; Rine and Herskowitz, 1987). It was shown by Kennedy *et al*. (1994) that deletion of *SIR2, SIR3* or *SIR4* results in shortened lifespan. The role of Sir proteins in ageing was further supported by studies on the *SIR4-42* mutation which increased lifespan by 50% (Kennedy *et al.*, 1995).

Phenotypes associated with yeast ageing include cell enlargement, nucleolar enlargement and fragmentation, relocalisation of the Sir complex to the nucleolus, and sterility (Sinclair *et al.*, 1997). Sinclair and Guarente (1997) showed that extrachromosomal rDNA circles (ERCs) excise from the rDNA locus in the nucleolus and replicate due to the presence of an autonomous replicating sequence present in each repeat. Asymmetrical segregation of ERCs during cell division results in their accumulation in ageing mother cells. This accumulation provides the structural basis for the expanded and fragmented nucleoli observed in aged cells. The authors suggested that ERCs may cause ageing, and eventually death, by an unbalanced expression of one or more of the encoded RNAs or titration of essential replication or transcription factors that could result in the inability to replicate or transcribe genomic DNA.

Sinclair and Guarente (1997) suggested that the relocalisation of Sir3p and Sir4p to the nucleolus may be responsible for delayed ageing. Sir2p has been shown to repress rDNA recombination (Gottlieb and Esposito, 1989) and together with Sir3p and Sir4p, the complex delays ageing by blocking the accumulation of ERCs. Low intensity stresses that extend yeast lifespan include mild heat, increased salt, or the yeast equivalents of CR – low levels of amino acids or glucose (Anderson *et al.*, 2003; Bitterman *et al.*, 2003; Jiang *et al.*, 2000; Lin *et al.*, 2000). All of the aforementioned stresses induce *PNC1* (**Figure 2.5**). It encodes a nicotinamidase that depletes nicotinamide (Anderson *et al.*, 2003), a product inhibitor of Sir2p (Bitterman *et al.*, 2002; Gallo *et al.*, 2004). Alternatively, Lin *et al*. (2004) propose activation of Sir2p is mediated by a decrease in cellular NADH, a competitive inhibitor of Sir2p. Nonetheless, enhanced Sir2p activity results in silencing and increased stability of rDNA (Lin *et al.*, 2000), thus mediating lifespan extension.

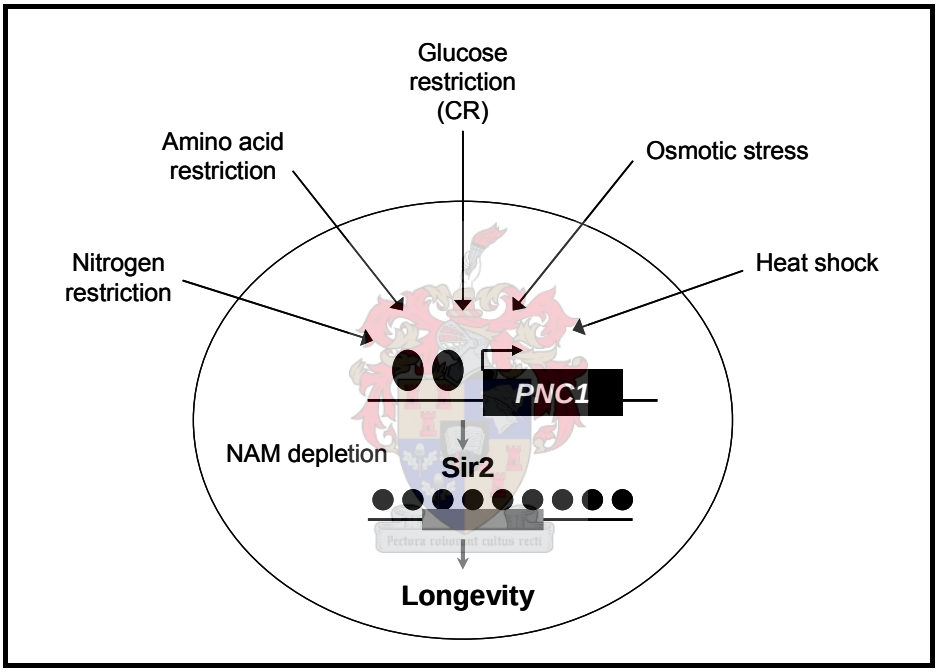


Figure 2.5. Replicative lifespan extension by caloric restriction mediated by Sir2p (Sinclair, 2005).

2.5.3.1.1 Resveratrol, modulator of sirtuin activity

Howitz *et al.* (2003) demonstrated that resveratrol was able to stimulate Sir2p activity twofold and it extended average yeast RLS by 70%. That glucose limitation, a form of CR, did not extend the lifespan of resveratrol-treated cells indicated that resveratrol and CR most probably act through the same pathway. The authors showed that resveratrol acts downstream of *PNC1* in a *SIR2* dependent manner. The authors propose that the ability of organisms to respond to stress molecules produced by plants has been retained or developed during evolution to enable them to prepare for adverse conditions. These

results form part of the substantiation of the 'xenohormesis' hypothesis by Howitz and Sinclair (Howitz *et al.*, 2003; Lamming *et al.*, 2004).

Subsequent to the study by Howitz *et al*. (2003), the scope for the search of potential CR mimetics that could have medicinal applications has broadened significantly. Previously, research focus was mainly on compounds that could modulate energy metabolism but has been extended to molecules that boost the activity of longevity regulators, possibly with no or fewer side effects (Sinclair, 2005).

2.5.3.2 Sir2p-independent model

Fundamental to these ageing studies is Fob1p, the replication fork barrier protein. Fob1p is required for most rDNA recombination and the generation of ERCs (Defossez *et al.*, 1999). Deletion of *FOB1* suppresses hyper-recombination in the rDNA and the short lifespan of *sir2*Δ mutants (Kaeberlein *et al.*, 1999).

Kaeberlein *et al*. (2004) propose that the pathway by which CR enhances lifespan is independent of Sir2p. Evidence supporting this is as follows:

- The combination of CR and *SIR2* overexpression results in an additive lifespan increase, consistent with the expectation of two genetic interventions acting in parallel pathways.
- In a *fob1*Δ mutant, CR results in a larger relative increase in lifespan in the absence of Sir2p than in cells where Sir2p is expressed.
- The ability of CR to promote longevity in a sir2Δ mutant.

The authors do not completely reject previous findings by other research groups, but they suggest that the role of Sir2p in CR mediated lifespan extension is minor. At least two pathways that regulate ageing in yeast were proposed: one is ERC accumulation and the other is responsive to CR (ERC independent), although undefined as yet at the molecular level (**Figure 2.6**). Kaeberlein *et al*. (2004) argue that the large body of evidence supporting the Sir2p-dependent model was generated using an unusual yeast strain, PSY316 and the findings are not consistent when tested in other yeast strains. Intense debate exists over strains used and techniques employed to induce calorie restriction and overall, does not shed much light on the rationalisation of contradictory findings (Lamming *et al.*, 2006).

The lifespan extending effects of resveratrol on yeast (Howitz *et al.*, 2003) could also not be reproduced by Kaeberlein *et al.* (2005), even after ruling out the possibility of a strain-dependent effect of resveratrol. They concluded that rather than being a general activator of sirtuins, resveratrol specifically stimulates Sir2 orthologs, in a substrate specific manner. This would account for their lack of phenotypes observed in yeast cells cultured in the presence of resveratrol.

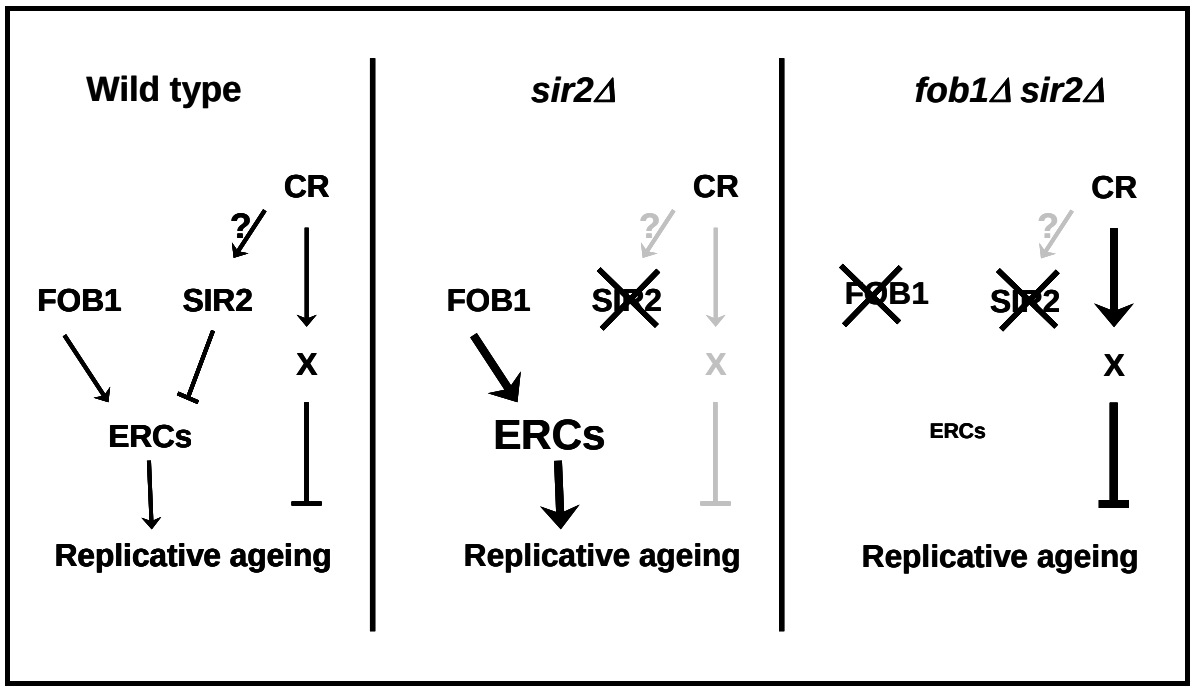


Figure 2.6. Two proposed pathways regulate yeast longevity mediated either by altered ERC levels or CR. In cells lacking Sir2p but containing Fob1p, senescence due to ERCs predominates, causing a shortened lifespan that cannot be increased by CR. In cells lacking FOB1, ERCs are greatly reduced and the CR pathway predominates. The presence or absence of Sir2p does not influence the longevity benefits of CR under this condition (Kaeberlein *et al.*, 2004).

2.6 CONCLUSION

Examining numerous aspects of a compound from different perspectives provides a good basis for identifying novel areas for further research in order to gain a more complete understanding of the inherent processes present in an organism, and how the compound affects the functioning thereof, if at all. Investigating the role resveratrol plays in the ageing of yeast is a good example. It has opened the field of ageing research to consider the existence of other pathways responsible for ageing besides the ones originally identified as a result of resveratrol's involvement.

The many guises of resveratrol result in highly complex interactions within the context of each organism discussed. Consequently, the likelihood of attaining a simple answer, whatever the question is therefore not good. A background into the origin of resveratrol and its derivatives together with their functioning as antifungal compounds in plants was presented. Interestingly, for many years the focus of research into stilbene levels in wine, and the factors affecting them, has been almost exclusively on *trans*-resveratrol, despite the evidence of its modification *in planta*. As more evidence is gathered on the absorption and bioavailability of resveratrol in humans, the idea of *trans*-resveratrol, as the singular compound being responsible for the health benefits derived from moderate wine consumption seems unlikely. More recently research focus has shifted towards evaluating the levels of resveratrol derivatives in wine, which gives a broader picture of the scope of

bioactive compounds present. Total stilbene concentrations measured in wine appear to come closer to being able to supply sufficient resveratrol in the diet to mediate effects observed *in vitro*. This is, of course, still subject to the absorption and bioavailability of these compounds *in vivo*. Thus, it seems more plausible that resveratrol, together with the range of other compounds present, could mediate the protection afforded by moderate wine consumption.

2.7 LITERATURE CITED

Adrian M, Jeandet P, Bessis R, Joubert JM. 1996. Induction of phytoalexin (resveratrol) synthesis in grapevine leaves treated with aluminum chloride ($AlCl_3$). *J Agric Food Chem* **44**: 1979-1981.

Adrian M, Jeandet P, Douillet-Breuil AC, Tesson L, Bessis R. 2000. Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. *J Agric Food Chem* **48**: 6103-6105.

Adrian M, Jeandet P, Veneau J, Weston LA, Bessis R. 1997. Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea*, the causal agent for gray mold. *J Chem Ecol* **23**: 1689-1702.

Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Sinclair DA. 2003. Nicotinamide and *PNC1* govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. *Nature* **423**: 181-185.

Arora A, Nair MG, Strasburg GM. 1998. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch Biochem Biophys* **356**: 133-141.

Artursson P, Karlsson J. 1991. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem Biophys Res Commun* **175**: 880-885.

Ashrafi K, Sinclair D, Gordon JI, Guarente L. 1999. Passage through stationary phase advances replicative aging in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **96**: 9100-9105.

Aumont V, Krisa S, Battaglia E, Netter P, Richard T, Merillon JM, Magdalou J, Sabolovic N. 2001. Regioselective and stereospecific glucuronidation of *trans*- and *cis*-resveratrol in human. *Arch Biochem Biophys* **393**: 281-289.

Becker JVW, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* **4**: 79-85.

Belguendouz L, Fremont L, Linard A. 1997. Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochem Pharmacol* **53**: 1347-1355.

Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. 2002. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem* **277**: 45099-45107.

Bitterman KJ, Medvedik O, Sinclair DA. 2003. Longevity regulation in *Saccharomyces cerevisiae*: linking metabolism, genome stability, and heterochromatin. *Microbiol Mol Biol Rev* **67**: 376-399.

Borie B, Jeandet P, Parize A, Bessis R, Adrian M. 2004. Resveratrol and stilbene synthase mRNA production in grapevine leaves treated with biotic and abiotic phytoalexin elicitors. *Am J Enol Vitic* **55**: 60-64.

Breuil AC, Adrian M, Pirio N, Meunier P, Bessis R, Jeandet P. 1998. Metabolism of stilbene phytoalexins by *Botrytis cinerea*: 1. Characterization of resveratrol dehydrodimer. *Tetrahedron Lett* **39**: 537-540.

Brill SS, Furimsky AM, Ho MN, Furniss MJ, Li Y, Green AG, Bradford WW, Green CE, Kapetanovic IM, Iyer LV. 2006. Glucuronidation of trans-resveratrol by human liver and intestinal microsomes and UGT isoforms. *J Pharm Pharmacol* **58**: 469-479.

Bryk M, Banerjee M, Murphy M, Knudsen KE, Garfinkel DJ, Curcio MJ. 1997. Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev* **11**: 255-269.

Calderon AA, Zapata JM, Pedreno MA, Munoz R, Ros Barcelo A. 1992. Levels of 4-hydroxystilbene-oxidizing isoperoxidases related to constitutive desease resistance in *in-vitro*-cultured grapevine. *Plant Cell, Tissue Organ Cult* **29**: 63-70.

Cantos E, Espin JC, Fernandez MJ, Oliva J, Tomas-Barberan FA. 2003. Postharvest UV-C-irradiated grapes as a potential source for producing stilbene-enriched red wines. *J Agric Food Chem* **51**: 1208-1214.

Cantos E, Espín JC, Tomás-Barberán FA. 2002. Postharvest Stilbene-enrichment of red and white table grape varieties using UV-C irradiation pulses. *J Agric Food Chem* **50**: 6322-6329.
Castellari M, Spinabelli U, Riponi C, Amati A. 1998. Influence of some technological practices on the quantity of resveratrol in wine. *Z Lebensm-Unters Forsch* **206**: 151-155.
Chiron H, Drouet A, Lieutier F, Payer HD, Ernst D, Sandermann H. 2000. Gene induction of stilbene synthase biosynthesis in Scots pine in response to ozone treatment, wounding, and fungal infection. *Plant Physiol* **124**: 865-872.
Clare SS, Imhoff S, Skurray GR. 2002. Effect of a pectolytic enzyme on the concentration of resveratrol isomers in wine. *Aust Grapegrower Winemaker* **461a**: 98-101.
Clare SS, Skurray G, Shalliker RA. 2004. Effect of pomace-contacting method on the concentration of *cis*- and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Am J Enol Vitic* **55**: 401-406.
Clare SS, Skurray GR, Shalliker RA. 2005. Effect of yeast strain selection on the concentration of *cis*- and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Aust J Grape Wine Res* **11**: 9-14.
Creasy LL, Coffee M. 1988. Phytoalexin production potential of grape berries. *J Am Soc Hortic Sci* **113**: 230-234.
Dammann R, Lucchini R, Koller T, Sogo JM. 1993. Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res* **21**: 2331-2338.
Daniels LB, Coyle PJ, Chiao YB, Glew RH, Labow RS. 1981. Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver. *J Biol Chem* **256**: 13004-13013.
Day AJ, Canada FJ, Diaz JC, Kroon PA, McLauchlan R, Faulds CB, Plumb GW, Morgan MR, Williamson G. 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett* **468**: 166-170.
Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJC, Morgan MRA, Williamson G. 1998. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Lett* **436**: 71-75.
De Santi C, Pietrabissa A, Spisni R, Mosca F, Pacifici GM. 2000. Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. *Xenobiotica* **30**: 609-617.
Defossez PA, Prusty R, Kaeberlein M, Lin SJ, Ferrigno P, Silver PA, Keil RL, Guarente L. 1999. Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol Cell* **3**: 447-455.
Delcroix A, Gunata Z, Sapis J-C, Salmon J-M, Bayonove C. 1994. Glycosidase activities of three enological yeast strains during winemaking: effect on the terpenol content of muscat wine. *Am J Enol Vitic* **45**: 291-296.
Dercks W, Creasy LL. 1989. Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. *Physiol Mol Plant Pathol* **34**: 203-213.
Doner LW, Bécard G, Irwin PL. 1993. Binding of flavonoids by polyvinylpolypyrrolidone. *J Agric Food Chem* **41**: 753-757.
Douillet-Breuil AC, Jeandet P, Adrian M, Bessis R. 1999. Changes in the phytoalexin content of various *Vitis* spp. in response to ultraviolet C elicitation. *J Agric Food Chem* **47**: 4456-4461.
Dupont MS, Gee JM, Price KR, Johnson IT. 1999. The availability of flavonol glycosides for small intestinal transport. *Gut 44*: (Suppl 1), A130.
Ector BJ, Magee JB, Hegwood CP, Coign MJ. 1996. Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds, and wines. *Am J Enol Vitic* **47**: 57-62.
Fabrizio P, Gattazzo C, Battistella L, Wei M, Cheng C, McGrew K, Longo VD. 2005. Sir2 blocks extreme life-span extension. *Cell* **123**: 655-667.
Fabrizio P, Longo VD. 2003. The chronological life span of *Saccharomyces cerevisiae*. *Aging Cell* **2**: 73-81.
Fabrizio P, Pletcher SD, Minois N, Vaupel JW, Longo VD. 2004. Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in *Saccharomyces cerevisiae*. *FEBS Lett* **557**: 136-142.
Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD. 2001. Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292**: 288-290.
Felix R, Villettaz JC. 1983. Wine. In *Industrial enzymology the application of enzymes in industry,* Godfrey T and Reichelt J (eds). The Nature Press: New York; 410-421.
Franco M, Coloru G, Del Caro A, Emonti G, Farris G, Manca G, Massa T, Pinna G. 2002. Variability of resveratrol (3,5,4'-trihydroxystilbene) content in relation to the fermentation processes by *Saccharomyces cerevisiae* strains. *Eur Food Res Technol* **214**: 221-225.
Fremont L. 2000. Biological effects of resveratrol. *Life Sci* **66**: 663-673.
Fritze CE, Verschueren K, Strich R, Easton Esposito R. 1997. Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. *Embo J* **16**: 6495-6509.

Gallo CM, Smith DL, Jr., Smith JS. 2004. Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol Cell Biol* **24**: 1301-1312.

Gambelli L, Santaroni GP. 2004. Polyphenols content in some Italian red wines of different geographical origins. *J Food Comp Anal* **17**: 613-618.

Gambuti A, Strollo D, Ugliano M, Lecce L, Moio L. 2004. *trans*-Resveratrol, quercetin, (+)-catechin, and (-)-epicatechin content in South Italian monovarietal wines: relationships with maceration time and marc pressing during winemaking. *J Agric Food Chem* **52**: 5747-5751.

Goldberg DM. 1995. Does wine work? *Clin Chem* **41**: 14-16.

Goldberg DM, Ng E, Karumanchiri A, Diamandis EP, Soleas GJ. 1996. Resveratrol glucosides are important components of commercial wines. *Am J Enol Vitic* **47**: 415-420.

Goldberg DM, Ng E, Karumanchiri A, Yan J, Diamandis EP, Soleas GJ. 1995a. Assay of resveratrol glucosides and isomers in wine by direct-injection high-performance liquid chromatography. *J Chromatogr* **708**: 89-98.

Goldberg DM, Soleas GJ, Hahn SE, Diamandis EP, Karumanchiri A. 1997. Identification and assay of trihydroxystilbenes in wine and their biological properties. In *Wine: Nutritional and Therapeutic Benefits,* Watkins TR (ed.). American Chemical Society: Washington DC; 24-43.

Goldberg DM, Yan J, Ng E, Diamandis EP, Karumanchiri A, Soleas G, Waterhouse AL. 1995b. A Global Survey of Trans-Resveratrol Concentrations in Commercial Wines. *Am J Enol Vitic* **46**: 159-165.

Goldberg DM, Yan J, Soleas GJ. 2003. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem* **36**: 79-87.

Gopalan V, Pastuszyn A, Galey WR, Jr., Glew RH. 1992. Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic beta-glucosidase. *J Biol Chem* **267**: 14027-14032.

Gottlieb S, Esposito RE. 1989. A new role for a yeast transcriptional silencer gene, *SIR2*, in regulation of recombination in ribosomal DNA. *Cell* **56**: 771-776.

Gottschling DE, Aparicio OM, Billington BL, Zakian VA. 1990. Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* **63**: 751-762.

Grunstein M. 1998. Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell* **93**: 325-328.

Guarente L. 1997. Link between aging and the nucleolus. *Genes Dev* **11**: 2449-2455.

Guarente L, Kenyon C. 2000. Genetic pathways that regulate ageing in model organisms. *Nature* **408**: 255-262.

Harris N, Costa V, MacLean M, Mollapour M, Moradas-Ferreira P, Piper PW. 2003. Mnsod overexpression extends the yeast chronological (G(0)) life span but acts independently of Sir2p histone deacetylase to shorten the replicative life span of dividing cells. *Free Radic Biol Med* **34**: 1599-1606.

Harris N, MacLean M, Hatzianthis K, Panaretou B, Piper PW. 2001. Increasing *Saccharomyces cerevisiae* stress resistance, through the overactivation of the heat shock response resulting from defects in the Hsp90 chaperone, does not extend replicative life span but can be associated with slower chronological ageing of nondividing cells. *Mol Genet Genomics* **265**: 258-263.

Henry C, Vitrac X, Decendit A, Ennamany R, Krisa S, Merillon JM. 2005. Cellular uptake and efflux of *trans*-piceid and its aglycone *trans*-resveratrol on the apical membrane of human intestinal Caco-2 cells. *J Agric Food Chem* **53**: 798-803.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* **425**: 191-196.

Jannin B, Menzel M, Berlot J-P, Delmas D, Lancon A, Latruffe N. 2004. Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol* **68**: 1113-1118.

Jazwinski SM. 2002. Growing old: metabolic control and yeast aging. *Annu Rev Microbiol* **56**: 769-792.

Jeandet P, Adrian M, Breuil AC, Sbaghi M, Debord S, Bessis R, Weston LA, Harmon R. 2000. Chemical induction of phytoalexin synthesis in grapevines: Application to the control of grey mould (*Botrytis cinerea* Pers.) in the vineyard. *Acta Hortic* **528**: 591-596.

Jeandet P, Bessis R, Gautheron B. 1991. The production of resveratrol (3,5,4'-trihydroxystilbene) by grape berries in different developmental stages. *Am J Enol Vitic* **42**: 41-46.

Jeandet P, Bessis R, Meunier P. 1995a. Production of the phytoalexin resveratrol by grapes as a response to Botrytis attack under natural conditions. *J Phytopath* **143**: 135-139.

Jeandet P, Bessis R, Sbaghi M, Meunier P, Trollat P. 1995b. Resveratrol content of wines of different ages: Relationship with fungal disease pressure in the vineyard. *Am J Enol Vitic* **46**: 1-4.

Jeandet P, Breuil AC, Adrian M, Weston LA, Debord S, Meunier P, Maume G, Bessis R. 1997. HPLC analysis of grapevine phytoalexins coupling photodiode array detection and fluorometry. *Anal Chem* **69**: 5172-5177.

Jeandet P, Douillet Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M. 2002. Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *J Agric Food Chem* **50**: 2731-2741.

Jeandet P, Sbaghi M, Bessis R. 1993. *The significance of stilbene type phytoalexin degradation by culture filtrates of Botrytis cinerea in vine-Botrytis interaction*. Kluwer: Dordrecht, Nertherlands.

Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM. 2000. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *Faseb J* **14**: 2135-2137.

Jones DP, Delong MJ. 2000. Detoxification and protective functions of nutrients. In *Biochemical and physiological aspects of human nutrition,* Stipanuk MH (ed.). W.B. Saunders Co.: Philadelphia; 902-911.

Kaeberlein M, Kirkland KT, Fields S, Kennedy BK. 2004. Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol* **2**: 1381-1387.

Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A, Kennedy BK. 2005. Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem* **280**: 17038-17045.

Kaeberlein M, McVey M, Guarente L. 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev* **13**: 2570-2580.

Kaldas MI, Walle UK, Walle T. 2003. Resveratrol transport and metabolism by human intestinal Caco-2 cells. *J Pharm Pharmacol* **55**: 307-312.

Kennedy BK, Austriaco NR, Jr., Guarente L. 1994. Daughter cells of *Saccharomyces cerevisiae* from old mothers display a reduced life span. *J Cell Biol* **127**: 1985-1993.

Kennedy BK, Austriaco NR, Jr., Zhang J, Guarente L. 1995. Mutation in the silencing gene SIR4 can delay aging in *S. cerevisiae*. *Cell* **80**: 485-496.

Krasnow MN, Murphy TM. 2004. Polyphenol glucosylating activity in cell suspensions of grape (*Vitis vinifera*). *J Agric Food Chem* **52**: 3467-3472.

Krisa S, Larronde F, Budzinski H, Descendit A, Deffieux G, Mérillon JM. 1999. Stilbene production by *Vitis vinifera* cell suspension cultures: Methyljasmonate induction and ^{13}C biolabeling. *J Nat Prod* **62**: 1688-1690.

Kroemer HK, Klotz U. 1992. Glucuronidation of drugs. A re-evaluation of the pharmacological significance of the conjugates and modulating factors. *Clin Pharmacokinet* **23**: 292-310.

Kuhnle G, Spencer JP, Chowrimootoo G, Schroeter H, Debnam ES, Srai SK, Rice-Evans C, Hahn U. 2000. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem Biophys Res Commun* **272**: 212-217.

LaMarco KL, Glew RH. 1986. Hydrolysis of a naturally occurring beta-glucoside by a broad-specificity beta-glucosidase from liver. *Biochem J* **237**: 469-476.

Lamming DW, Latorre-Esteves M, Medvedik O, Wong SN, Tsang FA, Wang C, Lin S-J, Sinclair DA. 2006. Response to Comment on "HST2 Mediates SIR2-Independent Life-Span Extension by Calorie Restriction". *Science* **312**: 1312.

Lamming DW, Wood JG, Sinclair DA. 2004. Small molecules that regulate lifespan: evidence for xenohormesis. *Mol Microbiol* **53**: 1003-1009.

Lamuela-Raventos RM, Romero-Perez AI, Waterhouse AL, de la Torre-Boronatt MC. 1995. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceid isomers in Spanish red *Vitis vinifera* wines. *J Agric Food Chem* **43**: 281-283.

Lamuela-Raventos RM, Waterhouse AL. 1993. Occurrence of resveratrol in selected California wines by a new HPLC method. *J Agric Food Chem* **41**: 521-523.

Lancon A, Delma D, Osman H, Thenot JP, Jannin B, Latruffe N. 2004. Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem Biophys Res Commun* **316**: 1132-1137.

Landrault N, Larronde F, Delaunay JC, Castagnino C, Vercauteren J, Merillon JM, Gasc F, Cros G, Teissedre PL. 2002. Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development. *J Agric Food Chem* **50**: 2046-2052.

Langcake P. 1981. Disease resistance of *Vitis* spp. and the production of the stress metabolites resveratrol, ε-viniferin, α-viniferin and pterostilbene. *Physiol Plant Pathol* **18**: 213-226.

Langcake P, Pryce RJ. 1976. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiol Plant Pathol* **9**: 77-86.

Langcake P, Pryce RJ. 1977a. A new class of phytoalexins from grapevines. *Experientia* **33**: 151-152.

Langcake P, Pryce RJ. 1977b. Oxidative dimerization of 4-hydroxystilbenes *in vitro*: Production of a grapevine phytoalexin mimic. *J Chem Soc Commun* **1412**: 208-210.
Langcake P, Pryce RJ. 1977c. The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. *Phytochemistry* **16**: 1193-1196.
Lanzarini G, Pifferi PG. 1989. Enzymes in the fruit juice industry. In *Biotechnology applications in beverage production,* Cantarelli C and Lanzarini G (eds). Elsevier Science Publishers Ltd.: London; 189-222.
Lin SJ, Defossez PA, Guarente L. 2000. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* **289**: 2126-2128.
Lin SJ, Ford E, Haigis M, Liszt G, Guarente L. 2004. Calorie restriction extends yeast life span by lowering the level of NADH. *Genes Dev* **18**: 12-16.
Liswidowati F, Melchior F, Hohmann F, Schwer B, Kindl H. 1991. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. *Planta* **183**: 307-314.
Lithgow GJ. 2001. Hormesis - a new hope for ageing studies or a poor second to genetics? *Hum Exp Toxicol* **20**: 301-303.
Longo VD, Ellerby LM, Bredesen DE, Valentine JS, Gralla EB. 1997. Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. *J Cell Biol* **137**: 1581-1588.
MacLean M, Harris N, Piper PW. 2001. Chronological lifespan of stationary phase yeast cells; a model for investigating the factors that might influence the ageing of postmitotic tissues in higher organisms. *Yeast* **18**: 499-509.
Martinez-Ortega MV, Garcia-Parrilla MC, Troncoso AM. 2001. Changes in phenolic composition of wines submitted to *in vitro* dissolution tests. *Food Chem* **73**: 11-16.
Masoro EJ. 2000. Caloric restriction and aging: an update. *Exp Gerontol* **35**: 299-305.
Masoro EJ, Austad SN. 1996. The evolution of the antiaging action of dietary restriction: a hypothesis. *J Gerontol A Biol Sci Med Sci* **51**: B387-91.
Mattivi F, Reniero F, Korhammer S. 1995. Isolation, characterisation, and evolution in red wine vinification of resveratrol monomers. *J Agric Food Chem* **43**: 1820-1823.
Mattson MP, Chan SL, Duan W. 2002. Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. *Physiol Rev* **82**: 637-672.
Mayer A, Harel E. 1979. Polyphenol oxidases in plants. *Phytochemistry* **18**: 193-215.
McMurrough I, Roche GP, Hennigan GP. 1984. Effect of PVPP dosage on the flavonoid contents of beer and the consequences for beer quality. *Brew Dig* **59**: 28-31.
Mellor JD, Layne DS. 1971. Steroid-D-glucosidase activity in rabbit tissues. *J Biol Chem* **246**: 4377-4380.
Mortimer RK, Johnston JR. 1959. Life span of individual yeast cells. *Nature* **183**: 1751-1752.
Okuda T, Yokotsuka K. 1996. *trans*-Resveratrol concentrations in berry skins and wines from grapes grown in Japan. *Am J Enol Vitic* **47**: 93-99.
Orallo F. 2006. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. *Curr Med Chem* **13**: 87-98.
Pannala AS, Chan TS, O'Brien PJ, Rice-Evans CA. 2001. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochem Biophys Res Commun* **282**: 1161-1168.
Pezet R, Gindro K, Viret O, Richter H. 2004a. Effects of resveratrol, viniferins and pterostilbenes on *Plasmopara viticola* zoospore mobility and disease development. *Vitis* **43**: 145-148.
Pezet R, Gindro K, Viret O, Spring JL. 2004b. Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. *Physiol Mol Plant Pathol* **65**: 297-303.
Pezet R, Perret C, Jean-Denis JB, Tabacchi R, Gindro K, Viret O. 2003. δ-Viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves. *J Agric Food Chem* **51**: 5488-92.
Pezet R, Pont V. 1990. Ultrastructural observations of pterostilbene fungitoxicity in dormant conidia of *Botrytis cinerea* Pers. *J Phytopath* **129**: 29-30.
Pezet R, Pont V, Hoang-Van K. 1991. Evidence for oxidative detoxication of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. *Physiol Mol Plant Pathol* **39**: 441-450.
Pryce RJ, Langcake P. 1977. α-Viniferin: An antifungal resveratrol trimer from grapevines. *Phytochemistry* **16**: 1452.
Rattan SI. 1998. Repeated mild heat shock delays ageing in cultured human skin fibroblasts. *Biochem Mol Biol Int* **45**: 753-759.
Rattan SI. 2004. Aging, anti-aging, and hormesis. *Mech Ageing Dev* **125**: 285-289.
Regev-Shoshani G, Shoseyov O, Bilkis I, Kerem Z. 2003. Glycosylation of resveratrol protects it from enzymic oxidation. *Biochem J* **374**: 157-163.

Rine J, Herskowitz I. 1987. Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. *Genetics* **116**: 9-22.
Robb DA. 1984. Tyrosinase. In *Copper Proteins and Copper Enzymes,* 2, Lontie R (ed.). CRC Press: Boca Raton; 207-241.
Ros Barcelo A, Pomer R, Lopez-Serrano M, Pedreno MA. 2003. Peroxidase: a multifunctional enzyme in grapevine. *Functional Plant Biol* **30**: 577-591.
Sacchi KL, Bisson LF, Adams DO. 2005. A review of the effect of winemaking techniques on phenolic extraction in red wines. *Am J Enol Vitic* **56**: 197-206.
Sakkiadi AV, Stavrakakis MN, Haroutounian SA. 2001. Direct HPLC assay of five biologically interesting phenolic antioxidants in varietal Greek red wines. *Lebensm-Wiss Technol* **34**: 410-413.
Schubert R, Fischer R, Hain R, Schreier PH, Bahnweg G, Ernst D, Sandermann H. 1997. An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. *Plant Mol Biol* **34**: 417-426.
Shaw PJ, Jordan EG. 1995. The nucleolus. *Annu Rev Cell Dev Biol* **11**: 93-121.
Siemann EH, Creasy LL. 1992. Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic* **43**: 49-52.
Sinclair DA. 2005. Toward a unified theory of caloric restriction and longevity regulation. *Mech Ageing Dev* **126**: 987-1002.
Sinclair DA, Guarente L. 1997. Extrachromosomal rDNA circles – a cause of aging in yeast. *Cell* **91**: 1033-1042.
Sinclair DA, Mills K, Guarente L. 1997. Accelerated aging and nucleolar fragmentation in yeast *sgs1* mutants. *Science* **277**: 1313-1316.
Smith JS, Boeke JD. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes Dev* **11**: 241-254.
Soleas GJ, Goldberg DM, Kaumanchiri A, Diamandis EP, Ng E. 1995. Influence of viticulture and oenological factors on changes in *cis*- and *trans*-resveratrol in commercial wines. *J Wine Res* **6**: 107-121.
Soleas GJ, Yan J, Goldberg DM. 2001a. Measurement of *trans*-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. *Methods Enzymol* **335**: 130-145.
Soleas GJ, Yan J, Goldberg DM. 2001b. Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. *J Chromatogr B Biomed Sci Appl* **757**: 161-172.
Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera L.*). *Plant Mol Biol* **24**: 743-755.
Sperker B, Backman JT, Kroemer HK. 1997. The role of β-glucuronidase in drug disposition and drug targeting in humans. *Clin Pharmacokinet* **33**: 18-31.
Threlfall RT, Morris JR (1996). Effect of viticultural and enological methods on the resveratrol content of wines. *Cool Climate Symposium for Viticulture and Oenology*. *New York*, pp. 100-102.
Turturro A, Hass B, Hart RW. 1998. Hormesis - implications for risk assessment caloric intake (body weight) as an exemplar. *Hum Exp Toxicol* **17**: 454-459.
Turturro A, Hass BS, Hart RW. 2000. Does caloric restriction induce hormesis? *Hum Exp Toxicol* **19**: 320-329.
Vacca V, Leccis L, Fenu P, Pretti L, Farris GA. 1997. Wine yeasts and resveratrol content. *Biotechnol Lett* **19**: 497-498.
Vaupel JW, Carey JR, Christensen K, Johnson TE, Yashin AI, Holm NV, Iachine IA, Kannisto V, Khazaeli AA, Liedo P, Longo VD, Zeng Y, Manton KG, Curtsinger JW. 1998. Biodemographic trajectories of longevity. *Science* **280**: 855-860.
Vickery ML, Vickery B. 1981. *Secondary Plant Metabolism*. The Macmillan Press Ltd: London.
Vitrac X, Bornet A, Vanderlinde R, Valls J, Richard T, Delaunay JC, Merillon JM, Teissedre PL. 2005. Determination of stilbenes (δ–viniferin, *trans*-astringin, *trans*-piceid, *cis*- and *trans*-resveratrol, ε–viniferin) in Brazilian wines. *J Agric Food Chem* **53**: 5664-5669.
Vitrac X, Desmouliere A, Brouillaud B, Krisa S, Deffieux G, Barthe N, Rosenbaum J, Merillon JM. 2003. Distribution of ^{14}C-*trans*-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci* **72**: 2219-2233.
Voragen AGJ, van den Broek LAM. 1991. Fruit juices. In *Biotechnological innovations in food processing,* Green J (ed.). Butterworth-Heinemann Ltd.: Oxford; 187-210.

Vrhovsek U, Wendelin S, Eder R. 1997. Effects of various vinification techniques on the concentration of *cis*- and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Am J Enol Vitic* **48**: 214-219.

Waffo-Teguo P, Decendit A, Vercauteren J, Deffieux G, Merillon J-M. 1996. *trans*-Resveratrol-3-O-β-glucoside (piceid) in cell suspension cultures of *Vitis vinifera*. *Phytochemistry* **42**: 1591-1593.

Waffo-Teguo P, Fauconneau B, Deffieux G, Huguet F, Vercauteren J, Merillon JM. 1998. Isolation, identification, and antioxidant activity of three stilbene glucosides newly extracted from *Vitis vinifera* cell cultures. *J Nat Prod* **61**: 655-657.

Waffo-Teguo P, Hawthorne ME, Cuendet M, Merillon JM, Kinghorn AD, Pezzuto JM, Mehta RG. 2001a. Potential cancer-chemopreventive activities of wine stilbenoids and flavans extracted from grape (*Vitis vinifera*) cell cultures. *Nutr Cancer* **40**: 173-179.

Waffo-Teguo P, Lee D, Cuendet M, Merillon J, Pezzuto JM, Kinghorn AD. 2001b. Two new stilbene dimer glucosides from grape (*Vitis vinifera*) cell cultures. *J Nat Prod* **64**: 136-138.

Warner JR. 1990. The nucleolus and ribosome formation. *Curr Opin Cell Biol* **2**: 521-527.

Waterhouse AL, Lamuela-Raventos RM. 1994. The occurrence of piceid, a stilbene glucoside, in grape berries. *Phytochemistry* **37**: 571-573.

Wiese W, Vornam B, Krause E, Kindl H. 1994. Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. *Plant Mol Biol* **26**: 667-677.

Wightman JD, Price SF, Watson BT, Wrolstad RE. 1997. Some effects of processing enzymes on anthocyanins and phenolics in Pinot noir and Cabernet Sauvignon wines. *Am J Enol Vitic* **48**: 39-48.

Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, Hirschelman WH, Pezzuto JM, Mehta RG, van Breemen RB. 2002. Human, rat, and mouse metabolism of resveratrol. *Pharm Res* **19**: 1907-1913.

Zinser C, Jungblut T, Heller W, Seidlitz HK, Schnitzler JP, Ernst D, Jr HS. 2000. The effect of ozone in Scots pine (*Pinus sylvestris* L.): gene expression, biochemical changes and interactions with UV-B radiation. *Plant, Cell Environ* **23**: 975-982.

3. RESEARCH RESULTS

Optimisation of both resveratrol production in recombinant *Saccharomyces cerevisiae* and the required analytical tools

Kim Trollope[1], Marietjie Stander[2], Melané Vivier[1] and Maret du Toit[1]

[1]Institute for Wine Biotechnology, Stellenbosch University, Private Bag X1, Matieland, Stellenbosch, 7602, South Africa

[2]Central Analytical Facility, Stellenbosch University, Private Bag X1, Matieland, Stellenbosch, 7602, South Africa

Abstract

Resveratrol is a phenolic compound produced by grapevine, mainly in the leaves and grape berry skins, in response to fungal attack. This compound possesses antioxidant activity and has been shown to have positive effects on human health relating to cardiovascular diseases, some cancers and the inhibition of inflammation. Resveratrol has also been shown to increase lifespan of lower eukaryotes. The principal motivation behind this study is the development of a yeast capable of producing resveratrol during grape must fermentation to increase the resveratrol content of wine and improve the wholesomeness of the product. Initial work showed that introducing multiple copies of the genes encoding coenzyme A ligase (*4CL216*) from hybrid poplar and grapevine resveratrol synthase (*vst1*) into *Saccharomyces cerevisiae* enabled the yeast to produce resveratrol. The current study focused on the optimisation of resveratrol production by laboratory yeast strains. Constitutive expression of integrated copies of *vst1* and *CL216* in three different yeast genetic backgrounds was achieved as confirmed by Southern and Northern blot analyses. The extraction of resveratrol from yeast was optimised for the intracellular fraction. Detection and quantification of the yeast derived resveratrol was performed using LCMSMS. Results from both intra- and extracellular fractions facilitated the elucidation of the dynamics of resveratrol production in yeast. It was shown that yeast genetic background significantly influenced the production kinetics and yield of resveratrol. Substrate availability in the form of *p*-coumaric acid proved to be a factor limiting resveratrol yields. Under conditions optimised in this study resveratrol was produced at levels of 0.35 mg/l, more than two hundred times higher than previously reported levels of 0.0015 mg/l. Preliminary findings indicate that endogenously-produced resveratrol may increase yeast lifespan.

3.1 INTRODUCTION

Resveratrol is a phytoalexin produced in leaves and grape berries of grapevine in response to biotic (Dercks and Creasy, 1989; Langcake and Pryce, 1976) and abiotic stresses (Adrian *et al.*, 1996; Langcake and Pryce, 1977). In addition to its *in planta* functions, resveratrol possesses antioxidant (Fauconneau *et al.*, 1997; Frankel *et al.*, 1993), phytoestrogen (Bowers *et al.*, 2000; Gehm *et al.*, 1997), anti-inflammatory and anticancer properties (Dong, 2003; Jang *et al.*, 1997). Resveratrol, by virtue of its antioxidant, vasodilatory and platelet antiaggregatory properties and modulation of lipoprotein metabolism, mediates protection against coronary heart disease (Arichi *et al.*, 1982; Bradamante *et al.*, 2004; Delmas *et al.*, 2005; Frankel *et al.*, 1993; Hao and He, 2004; Kimura *et al.*, 1985; Olas and Wachowicz, 2005; Wu *et al.*, 2001;). Its anticarcinogenic activities are attributed to its ability to inhibit cellular events associated with tumour initiation, promotion and progression (Jang *et al.*, 1997). Resveratrol has also been shown to promote life extension in *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster* and *Nothobranchius furzeri* (Howitz *et al.*, 2003; Valenzano *et al.*, 2006; Wood *et al.*, 2004).

Large scale epidemiological studies have highlighted the low incidence of coronary heart disease in France and Mediterranean countries in comparison to other western countries, despite the intake of a high-fat diet, low levels of physical activity and widespread smoking. This phenomenon, known as the French paradox (Renaud and de Lorgeril, 1992), was attributed to the prolonged, moderate intake of particularly red wine. The specific mechanism by which the French paradox functions has not yet been fully elucidated. However, a large body of evidence strongly supports the view that resveratrol is one of the main compounds responsible for the protective effects of moderate wine consumption (Siemann and Creasy, 1992). Owing to its production mainly in grape berry skins (Creasy and Coffee, 1988) and the differences between white and red wine vinification techniques, resveratrol levels are generally higher in red wines than in whites. During red wine vinification fermentation occurs on the skins, which allows for the extraction of phenolic compounds, including resveratrol, into the wine. The skins are separated from the grape must prior to fermentation in white wine vinification, thus limiting the extraction of phenolics. In a global survey, levels of *trans*-resveratrol in white wines were reported to be below 0.1 mg/l while in red wines levels were as high as 12.3 mg/l (Goldberg *et al.*, 1995). A recent survey of South African wines found levels of *trans*-resveratrol in red wines to be comparable, although slightly lower, to the levels reported by Goldberg *et al*. (1995) (de Villiers *et al.*, 2005).

The overall aim of our research is the development of a wine yeast strain capable of resveratrol production during grape must fermentation. We propose that red and white wines produced with such a resveratrol-producing yeast will contain elevated levels of resveratrol and that added health benefits may be derived from their consumption. Initial

work by Becker *et al*. (2003) established that yeast expressing genes encoding coenzyme A ligase and resveratrol synthase, which form part of the phenylpropanoid pathway in plants, was able to produce resveratrol.

In this study, we optimised the expression of the genes required for resveratrol synthesis and further investigated the dynamics of resveratrol production in *S. cerevisiae*. The method of sample preparation to extract resveratrol from the intracellular and extracellular fractions of yeast and the LCMSMS method for subsequent analysis were optimised. Results indicate that yeast genetic background influences yeast capability to synthesise resveratrol, and that precursor concentration is a limiting factor for maximal resveratrol production by yeast. An increase in yeast lifespan, possibly as a result of endogenously-produced resveratrol, was observed.

3.2 MATERIALS AND METHODS

3.2.1 MICROBIAL STRAINS, MEDIA AND CULTURE CONDITIONS

The bacterial and yeast strains, with their relevant genotypes, used in this study are listed in **Table 3.1**. All media components specified as percentages are given as weight per volume (w/v) unless otherwise stated. *Escherichia coli* served as host for the propagation of plasmids. Cultures were grown at 37°C in Luria Bertani (LB) broth (Biolab, Midrand, South Africa) containing 1.2% tryptone, 1.2% sodium chloride and 0.6% yeast extract. Solid media (LB agar) contained 1.5% agar. Where appropriate, media was supplemented with 100 μg/ml ampicillin (Roche, Mannheim, Germany) to allow for the selection of bacterial transformants.

Saccharomyces cerevisiae cultures were grown at 30°C in yeast peptone dextrose (YPD) broth (Biolab) containing 1.2% yeast extract, 2.5% peptone and 1.2% glucose. For auxotrophic selection of yeast transformants, synthetic complete (SC) media without either leucine or uracil, or neither leucine nor uracil was used. The media contained 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI, USA), supplemented with the drop-out amino acid pool (Rose *et al*., 1990). Solid media contained 1.5% agar (Difco).

3.2.2 DNA MANIPULATIONS AND PLASMID CONSTRUCTION

Standard procedures for the manipulation of DNA were used throughout the study (Sambrook *et al.*, 1989). Takara Ex Taq DNA polymerase (Takara BIO INC., Shiga, Japan), Supertherm DNA polymerase (Southern-Cross Biotechnology, Cape Town, South Africa), T4 DNA ligase (Fermentas, Vilnius, Lithuania) and restriction enzymes (Fermentas

and Roche) were used in the enzymatic manipulation of DNA according to the specifications of the suppliers.

Table 3.1. Microbial strains and plasmids used in this study.

Microbial strains	Genotype	Source
Bacterial strain		
Escherichia coli DH5α	F- φ80lacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 (rk-, mk+) supE44 thi-1 gyrA96 relA1 phoA	[a]GIBCO-BRL/Life Technologies
Yeast Strains		
S. cerevisiae FY23	*MATa leu2 trp1 ura3*	Winston *et al*. (1995)
S. cerevisiae CEN.PK2-1D	*MATα ura3-52 trp1-289 leu2-3_112 his3 D1 MAL2-8^C SUC2*	Entian and Kötter (1998)
S. cerevisiae Σ272	*MATα ura3-52 trp1△::hisG leu2△::hisG his3△::hisG*	This laboratory
FY23vst14CL216	*MATa LEU2 trp1 URA3 vst1 4CL216*	This study
FY23pRS305pCEL14	*MATa LEU2 trp1 URA3*	This study
CEN.PK2-1Dvst14CL216	*MATα URA3 trp1-289 LEU2 his3 D1 MAL2-8^C SUC2 vst1 4CL216*	This study
CEN.PK2-1DpRS305pCEL14	*MATα URA3 trp1-289 LEU2 his3 D1 MAL2-8^C SUC2*	This study
Σ272vst14CL216	*MATα URA3 trp1△::hisG LEU2 his3△::hisG vst1 4CL216*	This study
Σ272pRS305pCEL14	*MATα URA3 trp1△::hisG LEU2 his3△::hisG*	This study
Plasmids		
pVST1	Bluescript (f1 (IG) *rep LacZ bla*) *vst1*	Hain *et al*. (1993)
pUG64CL216	LoxP-*KanMX4*-LoxP *4CL216*	This laboratory
pRS305	Bluescript (f1 (IG) *rep LacZ bla*) *LEU2*	Sikorski and Hieter (1989)
pCel14	Ylp5 (Tc^R *bla URA3) PGK1$_P$ PGK1$_T$*	Gundllapalli *et al.* (2006)
pCEL14vst1	Ylp5 (Tc^R *bla URA3) PGK1$_P$ PGK1$_T$ vst1*	This study
pRS305vst1	Bluescript (f1 (IG) *rep LacZ bla*) *LEU2 PGK1$_P$ PGK1$_T$ vst1*	This study
pCEL144CL216	Ylp5 (Tc^R *bla URA3) PGK1$_P$ PGK1$_T$ 4CL216*	This study

[a]GIBCO/Bethesda Research Laboratories, Life Technologies Ltd., 3 Fountain Drive, Ichinnan Business Park, Paisley PA4 9RF.

The primers used in this study are listed in **Table 3.2**. The *vst1* gene encoding resveratrol synthase was amplified from pVST1 using the Vst1-F and Vst1-R primer pair.

After *Eco*RI and *Xho*I digestion of the PCR product, the 1187 base pair fragment was cloned into pCEL14 at the corresponding restriction enzyme sites. The correct orientation was confirmed by *Nde*I digestion. The resultant pCEL14vst1 contained the *vst1* expression cassette comprising *vst1* under the control of the 3-phosphoglycerate kinase (*PGK1*) promoter and terminator. The construct was verified by sequencing (Central Analytical Facility, Stellenbosch University). The *vst1* cassette was amplified from pCEL14vst1 with the PGK1-F and PGK1-R primers and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The *vst1* cassette was subcloned into pRS305 at the *Hind*III and *Bam*HI sites. This final construct was sequenced.

Table 3.2. Primers used in this study*.

Primer Name	Sequence (5' – 3')	RE site
Vst1-F	CC**GAATTC**CAATGGCTTCAGTCGAGGAA	*Eco*RI
Vst1-R	GCCG**CTCGAG**TTAATTTGTCACCATAGGAA	*Xho*I
Vst1p-F	TCTTGCAGAGAATAATGCAG	None
PGK1-F	CCC**AAGCTT**CTTTATTTTGGCTTCACCC	*Hind*III
PGK1-R	GC**GGATCC**GATAAATAATAGTCTATATATACG	*Bam*HI
4CL216-F	GATC**AGATCT**ATGGAGGCGAAAAATGATCA	*Bgl*II
4CL126-R	CT**CTCGAG**CTACTGCTGTTTTTGCATGTATG	*Xho*I
4CL216SDM-F	CTTGCAACTGGCGACTTCCTCATTAAATTC	None
4CL216p-F	CAGGTCTCCCTAAAGGTGTCA	None
4CL216p-R	GGACAAATGGCGCAATTGTCACCTTATA	None
ACT1-F	GACGCTCCTCGTGCTGTCTT	None
ACT1-R	GGAAGATGGAGCCAAAGCGG	None

* Restriction enzyme sites are indicated in bold.

In order to obtain a full length copy of *4CL216* it was necessary to remove a thymine (T) insert after position 1626 of the 1671 bp open reading frame. The insert introduced a premature stop codon and resulted in a 13 amino acid C-terminal truncation. PCR overlap extension mutagenesis (Higuchi *et al.*, 1988) was used to remove the T insert. Takara Ex Taq DNA polymerase (Takara BIO INC.) was used in the following reactions. In the first step of the mutagenesis, the reaction mixture contained 0.5 μg template DNA (pUG64CL216), 0.04 μM primers (4CL216SDM-F and 4CL216-R) and 50 μM dNTPs. The PCR conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 15 cycles of 20 s at 94°C, 40 s at 55°C, 10 s at 72°C, and a final extension of 3 min at 72°C. The product of the first reaction served as template DNA for the second step. A mixture of 20 μl of the reaction mixture from the first step, buffer and water was heated to 100°C for 3 min and allowed to cool gradually to 25°C. Subsequently, 50 μM dNTPs and 1 unit of DNA polymerase were added and incubated at 72°C for 3 min. Finally, 10 μM of 4CL216-F primer was added. The PCR cycles consisted of 1 min initial denaturation at

94°C, followed by 15 cycles of 20 s at 94°C, 40 s at 55°C, 1.5 min at 72°C, and the final elongation of 3 min at 72°C. The PCR product (approximately 1700 bp) was recovered from an agarose gel using the QIAGEN QIAquick gel extraction kit and ligated into pGEM-T Easy. Prior to bacterial transformation, the ligation mixture was digested with *Bst*EII and *Xba*I in order to linearise the original plasmid template. Restriction enzyme analysis and sequencing verified the construct. The *4CL216* fragment was isolated from pGEM-T Easy using *Bgl*II and *Xho*I and was subcloned into pCEL14 at the same sites. This final construct, pCEL144CL216 was confirmed by restriction analysis and sequencing.

3.2.3 YEAST TRANSFORMATION - SUCCESSIVE DOUBLE TRANSFORMATION

Yeast was transformed according to conventional methods (Gietz and Schiestl, 1995). The strains transformed are listed in **Table 3.1**. Yeast was initially transformed with pRS305vst1, linearised at the *Bst*EII site in *LEU2*. Putative transformants were selected on SC^{-leu} solid media. Following a PCR screen of genomic DNA with gene specific primers, positive transformants were subjected to a second round of transformation with pCEL144CL216 that was linearised with *Nco*I in *URA3*. Positive transformants were selected on $SC^{-ura-leu}$ solid media.

3.2.4 SOUTHERN HYBRIDISATION ANALYSIS

Southern hybridisation analysis using the digoxigenin non-radioactive nucleic acid labelling and detection system (Roche) was performed to confirm the integration of heterologous genes into the yeast genome. Yeast genomic DNA was prepared according to standard methods (Sambrook *et al.*, 1989). Genomic DNA was digested with *Cla*I when probing for *vst1* and *Hind*III when probing for *4CL216.* PCR generated DIG labelled probes were made using the Vst1p-F/Vst1-R and 4CL216p-F/4CL216p-R primer pairs, respectively. Reactions yielded a 650 bp probe for *vst1* and a 267 bp probe for *4CL216*. Lambda DNA digested with *Eco*RI and *Hind*III was used as a molecular weight marker. Hybridisation and signal detection was carried out according to the specifications of the manufacturer.

3.2.5 NORTHERN HYBRIDISATION ANALYSIS

Total RNA was extracted from yeast cells in the exponential growth phase (Ausubel *et al.*, 1994). Eight micrograms of RNA was subjected to denaturing gel electrophoresis and transferred to BioBond-Plus Nylon membranes (Sigma-Aldrich, Steinheim, Germany) according to standard procedures (Sambrook *et al.*, 1989). The same probes as described for the Southern hybridisation analysis were used for the northern hybridisation.

Hybridisation was carried out in high SDS buffer (7% SDS, 50% formamide, 4.5% skim milk powder, 0.1% *N*-lauroylsarcosine) at 50°C for 16 hours. Signal detection was performed according to the manufacturer's specifications. Transcript sizes were confirmed by comparison to a high range RNA ladder (Fermentas). To compensate for variations in amounts of RNA on the blot, normalisation was performed by comparing the signal strength of the heterologous genes to that of the actin gene (*ACT1*).

3.2.6 GROWTH CURVES AND YEAST VIABILITY COUNTS

YPD medium was inoculated with single yeast colonies and incubated overnight at 30°C. An appropriate volume of cells was transferred into fresh medium to reach an absorbance at 600 nm (OD_{600}) of 0.1 and grown overnight. Yeast cells from these cultures were subsequently used to inoculate rich medium, supplemented with a selected range of *p*-coumaric acid concentrations, to an initial OD_{600} of 0.1. The cultures were incubated at 30°C, shaking at 190 rpm. Four biological repeats were performed. Samples were taken approximately 3 hourly during the early growth stages, after which sampling periods were extended as growth rate decreased. Sample volume was controlled not to exceed 10% of the culture volume. Where appropriate, samples were diluted prior to taking OD_{600} readings. This ensured readings were obtained within the linear range of the spectrophotometer.

To determine yeast viability, samples were diluted appropriately in water and plated out on YPD solid media. Plates were incubated at 30°C for 2 days and colony forming units were enumerated.

3.2.7 RESVERATROL ASSAYS

3.2.7.1 Materials and standards

HPLC grade acetonitrile was obtained from Romil (Cambridge, UK), absolute ethanol from Merck (Darmstadt, Germany), ethyl acetate from Fluka (Buchs, Switzerland) and water was MilliQ grade passed through 0.22 μm filters. β-glucosidase from almonds was supplied by Sigma. *trans*-Resveratrol, naringenin and *p*-coumaric acid standards were purchased from Sigma. *trans*-Piceid (*E*-4,5'-dihyroxy-3'-glucopyranosylstilbene) was supplied by Polyphenols Laboratories AS (Sandnes, Norway). Naringenin was dissolved in absolute ethanol and added to samples prior to extraction.

3.2.7.2 Liquid chromatography tandem mass spectrometry (LCMSMS) method

The method previously described (Becker *et al.*, 2003) has been adapted and transferred to run on a Waters Quattro Micro mass spectrometer with Alliance 2690 HPLC. It entails

electrospray ionisation in the negative mode at a capillary voltage of 3.5 kV and MRM (multiple reaction monitoring) of the ions of interest. The rest of the MS settings were optimised for the highest sensitivity for resveratrol. The analysis method has been further developed to encompass the identification and quantification of naringenin, *p*-coumaric acid and *trans*-piceid. Separation was achieved on a Luna C18 (2), 2x150 mm, 3 µm column with an isocratic flow of 0.18 ml/min using 50% acetonitrile. The collision energy settings, cone voltages, retention times and parent and daughter ions that were monitored are given in **Table 3.3**.

Table 3.3. Details of LCMSMS method modifications.

Compound	Retention Time (min)	Cone Voltage	*m/z* of Parent ion	m/z of Daughter ion	Collision Energy
trans-Resveratrol	3.74	15	227	143	30
Naringenin	5.7	15	271	151	20
p-Coumaric acid	3.09	15	163	119	16
trans-Piceid	2.39	35	389	227	18

3.2.7.3 Yeast cultivation – resveratrol extraction from intracellular fraction of yeast

$SC^{-ura-leu}$ liquid medium was inoculated with a single yeast colony and shaken at 190 rpm for approximately 20 h. An appropriate volume of cells was transferred to fresh medium to give a resultant OD_{600} equal to 0.1. The culture was grown overnight. Yeast cells from this culture were subsequently used to inoculate 400 ml of SC liquid medium supplemented with 10 mg/l *p*-coumaric acid to an OD_{600} of 0.5. The flasks were shaken for 48 h at 30°C.

3.2.7.4 Resveratrol extraction procedure from intracellular fraction of yeast – liquid extraction

For each repeat 100 ml of cells was harvested. Extractions were done in triplicate. Cells were washed in water, pelleted and aliquoted into lysing matrix C tubes (Qbiogene, Montreal, Canada). Prior to cell disruption, the pellet was spiked with 200 ng naringenin which served as an internal standard for LCMSMS analyses. Cells were resuspended in an equal volume of either 80% or absolute ethanol and mechanically disrupted in the Savant FastPrep machine. Another volume of ethanol was added and extraction proceeded at 25°C or 60°C for 30 min. Following centrifugation, the supernatant was removed and dried either under vacuum or nitrogen. A second extraction step from the remaining cell pellet was repeated as described above.

The extract was treated with 0.25 units of β-glucosidase in 50 mM citrate phosphate buffer, pH 5.2 for 1 h at 37°C. Free resveratrol was subsequently extracted into acidified ethyl acetate. Following centrifugation, the organic phase was removed and dried under nitrogen. The extract was dissolved in 50% acetonitrile and analysed with LCMSMS.

3.2.7.5 Resveratrol extraction procedure from intracellular fraction of yeast - solid phase extraction

The cultivation, harvesting, cell disruption and extraction steps are the same as described for liquid extraction from the intracellular fraction of yeast. After centrifugation, the supernatant was recovered and made up to 1 ml with water to give a final concentration of 50% ethanol. Resveratrol was recovered by solid phase extraction as described previously (Gamoh and Nakashima, 1999). Briefly the method involved preconditioning the column with 5 ml methanol followed by 5 ml ammonium acetate (20 mM, pH 5.5). The sample was then applied to the column and washed with 2.5 ml methanol/20 mM ammonium acetate (pH 5.5) (10:90, v/v). Resveratrol was then eluted from the column with 1 ml methanol/20 mM ammonium acetate (pH 5.5) (85:15, v/v). The eluent was dried under vacuum, underwent β-glucosidase treatment and all subsequent steps as described above before being analysed by LCMSMS.

3.2.7.6 Yeast cultivation - extracellular resveratrol extraction

YPD medium was inoculated with a single yeast colony and shaken overnight at 30°C. An appropriate volume of cells was transferred to fresh medium to give an initial OD_{600} of 0.1 and the culture was grown overnight. Yeast cells from this culture were subsequently used to inoculate 500 ml of liquid medium, supplemented with 10 mg/l *p*-coumaric acid, to an OD_{600} of 0.1. Five biological repeats were performed. The flasks were shaken at 30°C at 190 rpm. Ten millilitre samples were removed at selected time points along the growth curve.

3.2.7.7 Extracellular resveratrol extraction procedure

Samples were centrifuged at 8000 rpm for 8 min. The cell pellets were discarded and supernatants kept at 4°C until extractions were performed. Extractions were performed within 24 hours of sampling. After spiking the supernatant with 200 ng of naringenin, extraction proceeded at 25°C for 30 min into 6 ml ethyl acetate. The organic phase was recovered and dried under nitrogen. The extract was treated with 0.25 units of β-glucosidase in 50 mM citrate phosphate buffer, pH 5.2 for 1 h at 37°C. Resveratrol was extracted into ethyl acetate and after centrifugation, the organic phase was recovered and dried under nitrogen. Samples were stored protected from light at -20°C until analysis by LCMSMS. Just prior to analysis, extracts were dissolved in 50% acetonitrile and filtered.

3.2.7.8 Yeast cultivation - precursor feeding study

Yeast harbouring the control plasmids was cultivated in rich media as described in section 3.2.8.6 supplemented with increasing concentrations of *p*-coumaric acid. *p*-Coumaric acid concentrations tested were 0, 5, 25, 50 and 100 mg/l. *p*-Coumaric acid was dissolved in absolute ethanol and used to spike the medium prior to inoculation. The starting concentration of ethanol was kept constant at 1%. After 48 h of growth, 10 ml of the supernatant was analysed for resveratrol and residual *p*-coumaric acid content.

3.3 RESULTS AND DISCUSSION

Three different genetic backgrounds of yeast (FY23, CEN.PK2-1D and Σ272) were transformed with genes encoding resveratrol synthase and coenzyme A ligase. In a proof-of-concept study conducted in our laboratory, we confirmed that yeast was able to produce resveratrol when transformed with multiple copies of the aforementioned genes and supplied with *p*-coumaric acid in the growth medium (Becker *et al.*, 2003). The levels of resveratrol detected were low in the initial study, leaving scope for improving the expression strategy, but also the extraction and analysis procedure from yeast cells. In the current study, we show that yeast containing single, integrated copies of the genes could produce considerable amounts of resveratrol. We elucidated the resveratrol-production kinetics of different yeast strains and showed that *p*-coumaric acid concentration is the limiting factor in resveratrol production at concentrations up to tenfold those previously tested.

3.3.1 INTEGRATION AND EXPRESSION OF THE HETEROLOGOUS GENES IN YEAST

Southern hybridisation analysis was used to confirm the integration of the relevant genes required for resveratrol synthesis into the yeast genome. Blots were probed separately for each introduced gene. Due to the nature of the genomic DNA digestion, single hybridisation signals were expected in positively integrated transformants and no hybridisation signals were expected in the yeast harbouring control plasmids pRS305 and pCEL14. When probing for *vst1* the expected band size was 3.9 kb and for *4CL216*, 8.5 kb. **Figure 3.1** shows the results of the hybridisation analyses - no signal was detected in the lanes marked N in hybridisations with either of the two probes, which correspond to yeast containing control plasmids only. Individual transformants tested were numbered T1-T3 in each respective genetic background transformed. T3 in the FY23 background; T1 and T2 in the CEN.PK2-1D background; and T1, T2 and T3 in the Σ272 background gave positive signals during hybridisation with the *vst1* probe. When probing

for *4CL216*, positive hybridisation signals were observed in all transformants of all three genetic backgrounds. The presence of an extra band present in some of the lanes could not be explained as there was no consistent pattern between genetic backgrounds, positive and negative transformants, nor was it present in the yeast harbouring control plasmids.

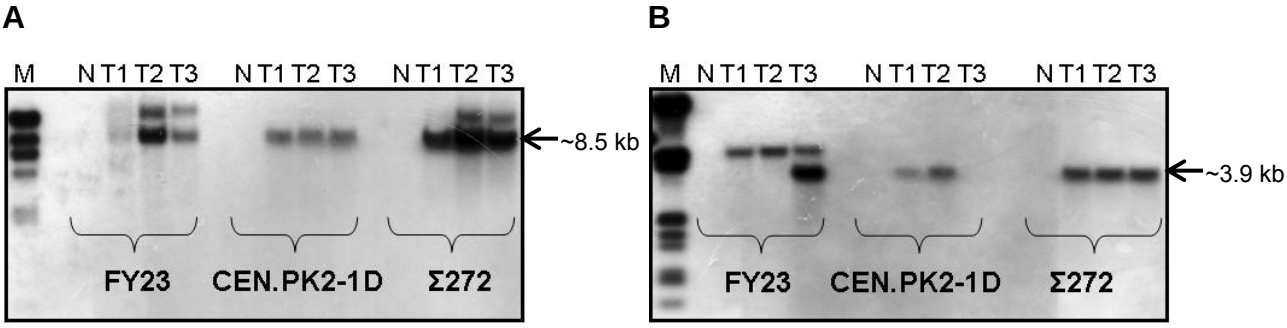


Figure 3.1. Southern hybridisation analyses confirming the integration of *4CL216* (A) and *vst1* (B) in the FY23, CEN.PK2-1D and Σ272 yeast genetic backgrounds. M denotes the marker (Lambda DNA digested with *Hind*III in A; *Eco*RI and *Hind*III in B), N the yeast harbouring control plasmids (pRS305 and pCEL14) and T1-T3 represent the individual transformants tested.

Northern hybridisation analysis of total RNA confirmed the expression of the integrated copies of *4CL216* and *vst1* in the 3 yeast backgrounds (**Figure 3.2**). The signal detected in lanes containing RNA isolated from positive transformants corresponded to the expected transcript size when compared to the High Range RNA ladder. As expected, positive transformants as determined by Southern hybridisation analysis gave positive signals in northern hybridisation, while no signal was detected for negative transformants. The exception being T1 in the FY23 background – Southern hybridisation analysis showed integration of *4CL216* but no corresponding signal was detected during hybridisation of the northern blot.

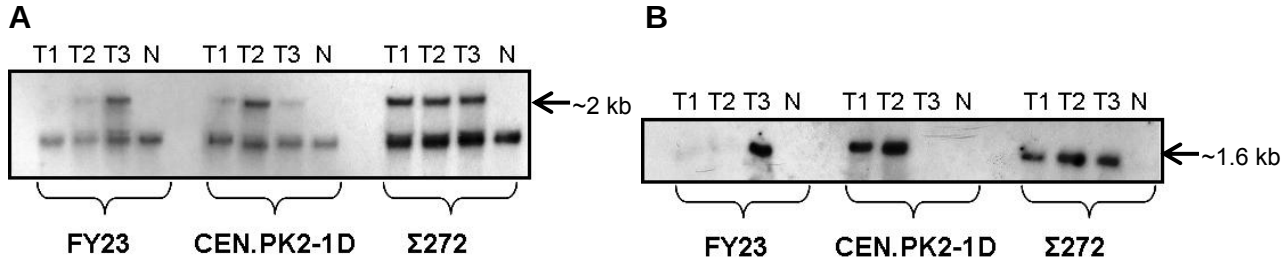


Figure 3.2. Northern hybridisation analyses of the strains transformed with *4CL216* and *vst1*. Total RNA was hybridised with DIG-labelled *4CL216* (A) or *vst1* (B) probes. In A the bottom band corresponds to the *ACT1* signal. N denotes the yeast harbouring control plasmids (pRS305 and pCEL14) and T1-T3 represent the individual transformants tested.

The results of the respective hybridisation analyses emphasise the importance of these steps when introducing genes into heterologous hosts. PCR analysis of the genomic DNA using gene specific primers (**Table 3.2**) indicated that all transformants contained both genes (results not shown). The detection of the heterologous genes by PCR does not confirm integration and similarly, positive integration of the genes does not

necessarily confirm that the genes will be expressed. **Table 3.4** summarises the results obtained from the Southern and northern hybridisation analyses and indicates the transformants selected for further analyses.

Table 3.4. Summary of results obtained from Southern and northern hybridisation analyses of yeast transformants.

Genetic background	Transformant with integrated copies of *vst1* and *4CL216*	Transformants expression *vst1* and *4CL216*	Transformant selected for analysis of resveratrol production
FY23	T3	T3	T3
CEN.PK2-1D	T1 T2	T1 T2	T2
Σ272	T1 T2 T3	T1 T2 T3	T1

3.3.2 RESVERATROL ASSAYS

The transformants, as indicated in **Table 3.4**, were analysed for resveratrol production. The assay involved the extraction of resveratrol from either the intracellular fraction of yeast or the growth medium, followed by LCMSMS analysis of the extracts for qualitative and quantitative determination of the resveratrol production by transformants. Becker *et al.* (2003) were the first to report the production of resveratrol in yeast and the method for extraction of resveratrol from yeast had not been optimised or described elsewhere in the literature. Sample preparation was therefore initially optimised prior to further analyses.

In all cases, no resveratrol could be detected in any intracellular and extracellular extracts from yeast transformed with the control plasmids (data not shown).

For extractions from the intracellular fraction of yeast, the starting inoculum for yeast cultivation was high (OD_{600} = 0.5) in order to obtain sufficient biomass for extraction. In assays where the supernatant was analysed, the initial inoculum for yeast cultivation was lower (OD_{600} = 0.1) in order to simulate a more typical yeast growth pattern.

3.3.2.1 LCMSMS method

In addition to resveratrol, the modifications to the published LCMSMS method (Becker *et al.*, 2003) allowed us to simultaneously detect naringenin, *p*-coumaric acid and *trans*-piceid. **Figure 3.3** shows chromatograms for the aforementioned compounds. Previously, Becker *et al*. (2003) reported that resveratrol could only be detected following β-glucosidase treatment of intracellular yeast extracts from yeast grown in minimal media

(the *trans*-picied standard was not available at that time). In this study, we found no difference in resveratrol concentrations between samples that were treated with β-glucosidase and untreated ones, which indicated that resveratrol was not glucosylated when yeast was grown in rich media and secreted by the cells. This result is confirmed by Beekwilder *et al.* (2006).

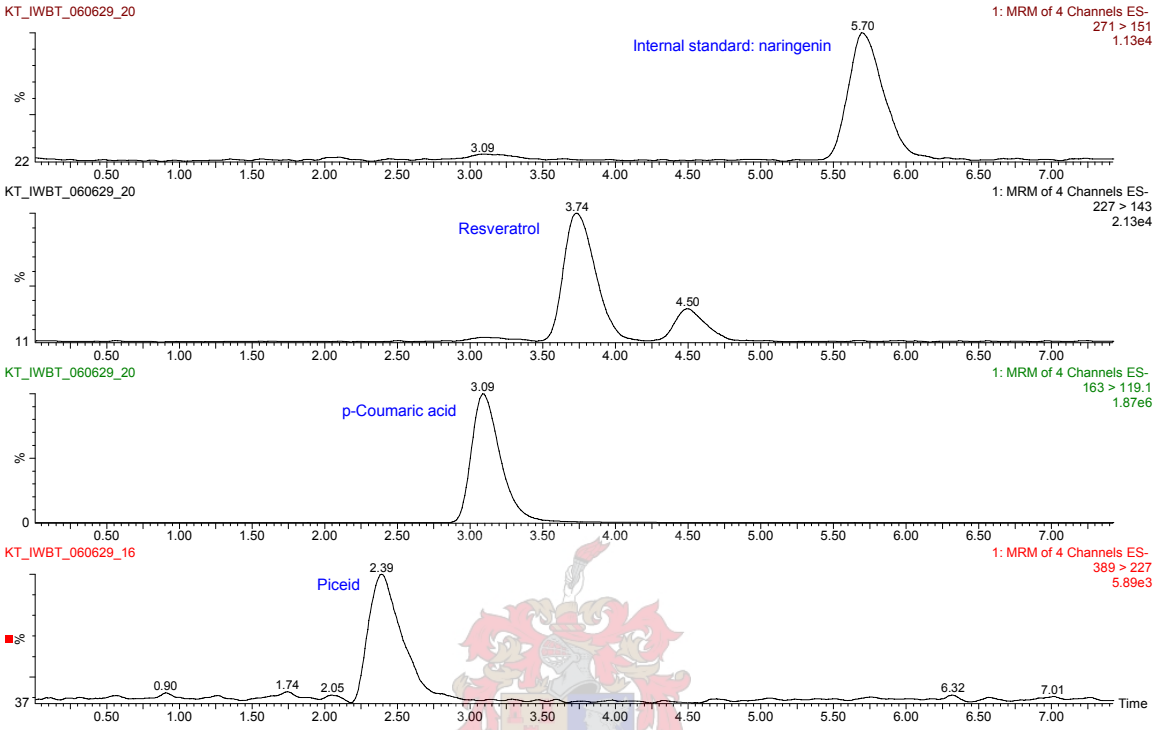


Figure 3.3. Chromatograms for naringenin, resveratrol, *p*-coumaric acid and *trans*-piceid. The extract analysed was taken from the supernatant of the CEN.PK2-1D transformant. The chromatogram for *trans*-piceid was obtained from analysing the commercially obtained compound.

Single peaks were obtained in the chromatograms for naringenin, *p*-coumaric acid and *trans*-piceid. Two peaks were, however, visible on the chromatogram for resveratrol which corresponded to the *trans*-isomer (large peak) and the *cis*-isomer (small peak) of resveratrol. We quantified only the *trans*-isomer as the *cis*-isomer for use as authentic standard is not commercially available. In addition, work by Becker *et al.* (unpublished, 2002) showed *cis*-resveratrol obtained from UV exposure of *trans*-resveratrol to be unstable.

3.3.2.2 Optimisation of the extraction of resveratrol from the intracellular fraction of yeast

In the previous study (Becker *et al*., 2003) extractions from the intracellular fractions were reported, thus initial work focused on the optimisation of the extraction of resveratrol from the intracellular fraction of yeast. Without the use of detergents which could interfere with LCMSMS analyses, disruption of yeast cells may be a limiting factor during extraction of

resveratrol from yeast. Numerous physical disruption methods were tested and included vortexing cells with glass beads, sonication, alternating freeze-thaw cycles and finally, vigorous shaking of cells (in tubes with glass beads containing an equal volume of ethanol to cells) in the Savant FastPrep machine proved most successful. Sufficient cell disruption was confirmed by microscopic examination.

Optimisation of the extraction of resveratrol and piceid isomers from grape berry skins indicated that the highest concentrations of these compounds were recovered in 80% ethanol at 60°C for 30 minutes (Romero-Perez *et al.*, 2001). As the optimisation of the extraction of resveratrol from yeast has not been described in the literature, preliminary testing employed these same conditions. Extractions were conducted at 25°C and 60°C to test the temperature sensitivity of resveratrol. Due to the viscous nature of the disrupted cell pellet, the necessity of a second extraction from the pellet was also investigated to determine whether this second extraction would improve the resveratrol yield. Results are shown in **Figure 3.4**. In addition to showing the optimal extraction parameters, the results indicate that yeast (FY23 genetic background) transformed with single copies of *vst1* and *4CL216* produced resveratrol in detectable and quantifiable amounts. Moreover, yeast cultivated in minimal media accumulated resveratrol intracellularly.

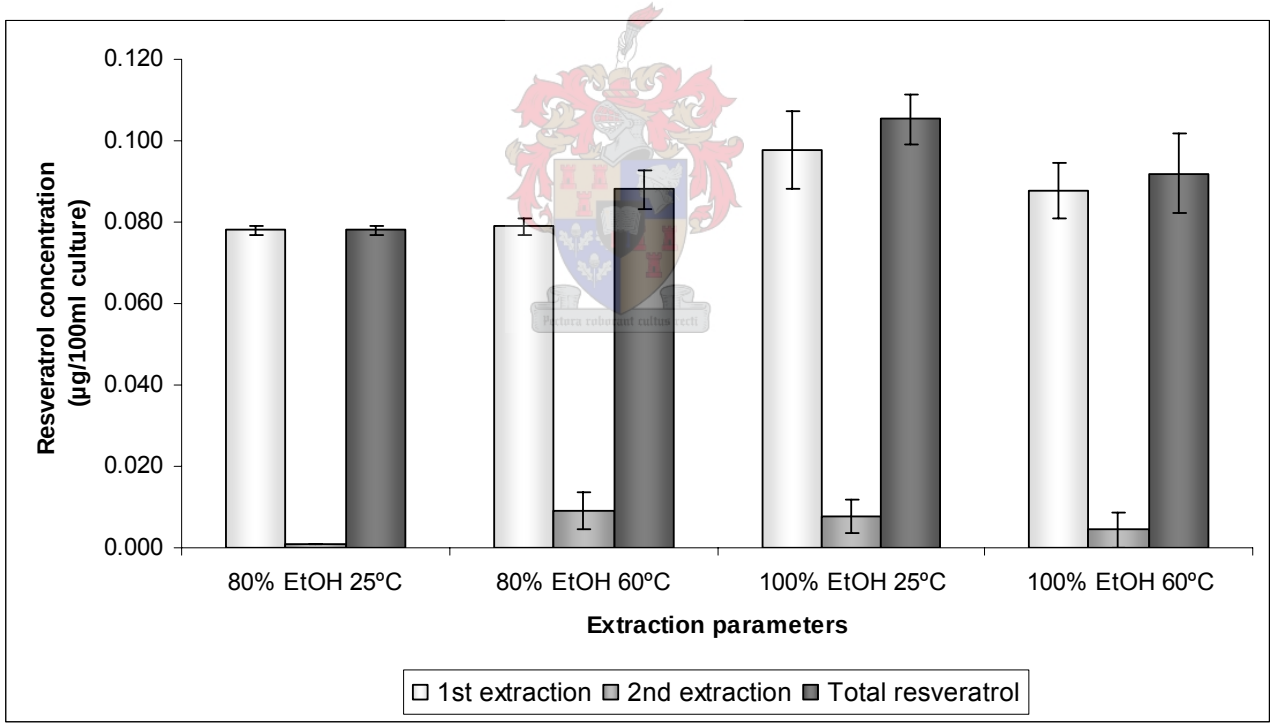


Figure 3.4. Influence of solvent concentration and temperature on the extraction of resveratrol from the intracellular fraction of transformed yeast grown in minimal media. The data are means ± standard error of the mean (n = 3).

Extraction into absolute ethanol at 25°C yielded the highest concentration of resveratrol (0.105 ug/100 ml culture). The second extraction from the cell pellet

contributed at most 11% (extraction into 80% ethanol at 60°C) of total resveratrol extracted. This step was not included in further experiments.

Conflicting results have been reported for resveratrol stability during sample drying under rotary evaporation (vacuum) (McMurtrey *et al.*, 1994; Pezet *et al.*, 1994; Trela and Waterhouse, 1996). Therefore, drying samples under vacuum was compared to drying under a stream of nitrogen. Results obtained indicated that there was no significant difference between resveratrol concentrations when samples were extracted into absolute ethanol and dried in either manner (**Figure 3.5**). However, when 80% ethanol was used as extraction solvent, samples dried under vacuum contained higher concentrations of resveratrol than those dried under nitrogen (0.088 µg/100 ml culture vs 0.066 µg/100 ml culture).

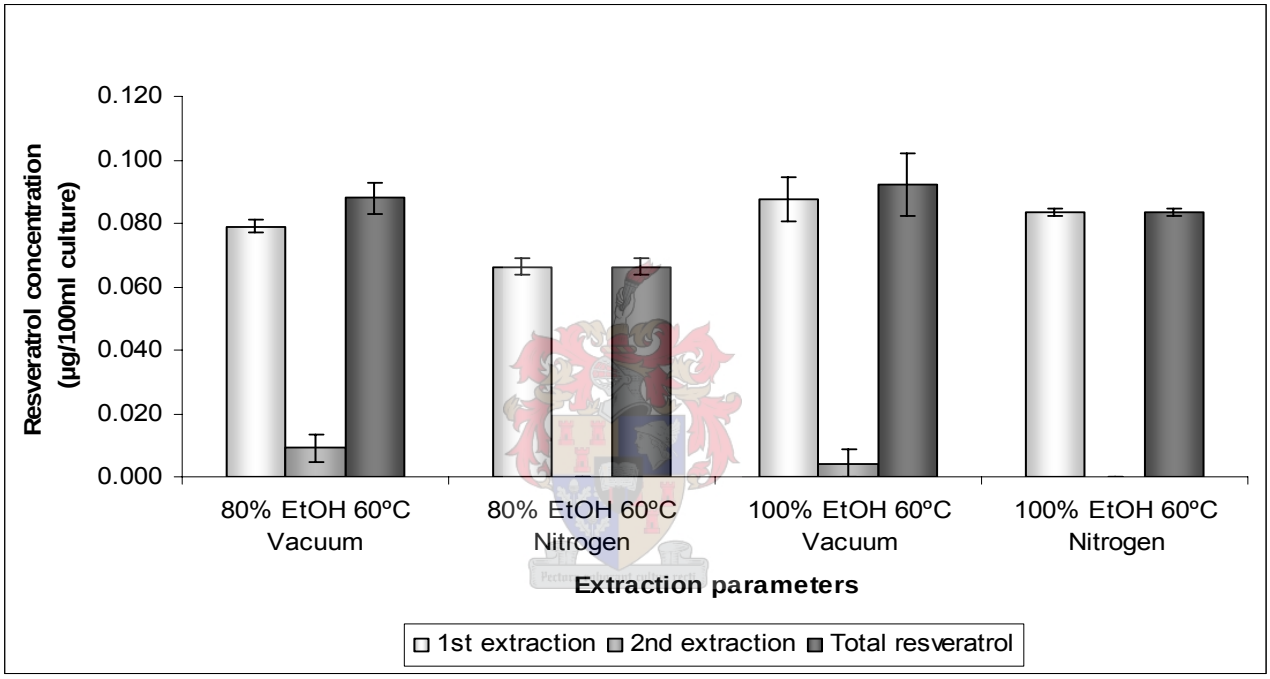


Figure 3.5. Comparison of two sample drying techniques during resveratrol extraction from transformed yeast grown in minimal media. The data are means ± standard error of the mean (n = 3).

Optimised conditions for the extraction of resveratrol from yeast cells could thus be summarised as follows: extraction in absolute ethanol as solvent at 25°C for 30 min was followed by drying of the extract under vacuum prior to enzymatic treatment. The final drying step was performed under nitrogen. Comparison of liquid and solid phase extraction showed no differences in the amount of resveratrol recovered (data not shown). However, the extract obtained via solid phase extraction was cleaner as it contained fewer compounds that were not of interest. The LCMSMS method employed selected ion monitoring (SIM), therefore the background noise was inconsequential. Further extractions were done without the solid phase cleanup step. Resveratrol recovery using the optimised extraction procedure was calculated to be 36%.

3.3.2.3 Supernatant extractions

When yeast was cultivated in rich media supplemented with *p*-coumaric acid, resveratrol was secreted by the cells. Intracellular and extracellular fractions from the three genetic backgrounds were tested at 12 and 48 hours post inoculation and resveratrol was only detected extracellularly (results not shown). Recovery of resveratrol from supernatant extractions was calculated to be 32% - data were not adjusted for recovery.

3.3.2.4 Growth curves

Growth patterns for transformed yeast strains of three different genetic backgrounds are shown in **Figure 3.6**. FY23 and CEN.PK2-1D were high biomass producers in comparison to Σ272. Growth of resveratrol-producing and non-producing strains appeared to follow a similar pattern. However, in the FY23 background significant differences in biomass production between non-producers and producing strains could be detected after 50 hours of growth. In a separate experiment, the same trend was seen in the FY23 background only (data not shown).

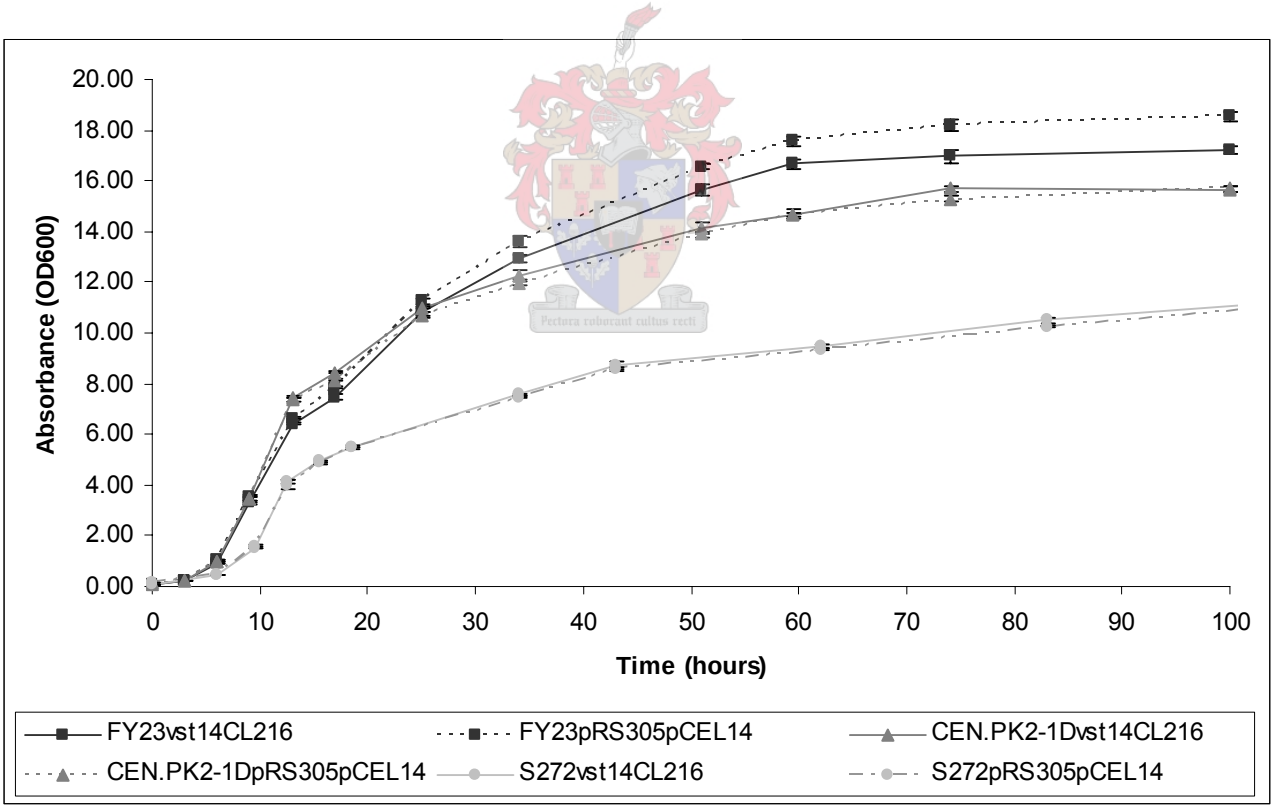


Figure 3.6. Growth, in rich media, of FY23, CEN.PK2-1D and Σ272 strains transformed with genes for resveratrol production and those harbouring control plasmids only. Data are mean ± standard error (n = 4). Solid lines represent resveratrol-producing transformants.

3.3.2.5 Time course assays

In order to elucidate the dynamics of resveratrol production in yeast, supernatants were analysed for resveratrol at time points corresponding to exponential, early and late stationary growth phases. The influence of yeast genetic background on resveratrol production was also investigated. Resveratrol was detected as early as 8 hours post inoculation with FY23, but resveratrol was only detected 16 hours post inoculation with Σ272 (**Figure 3.7**). Resveratrol production in both strains continued to increase up to 100 hours post inoculation, but FY23 was the superior producer. The large standard error can be ascribed to biological variation in resveratrol production within a genetic background. Σ272 appeared to be a more consistent producer as the standard error was proportionately smaller.

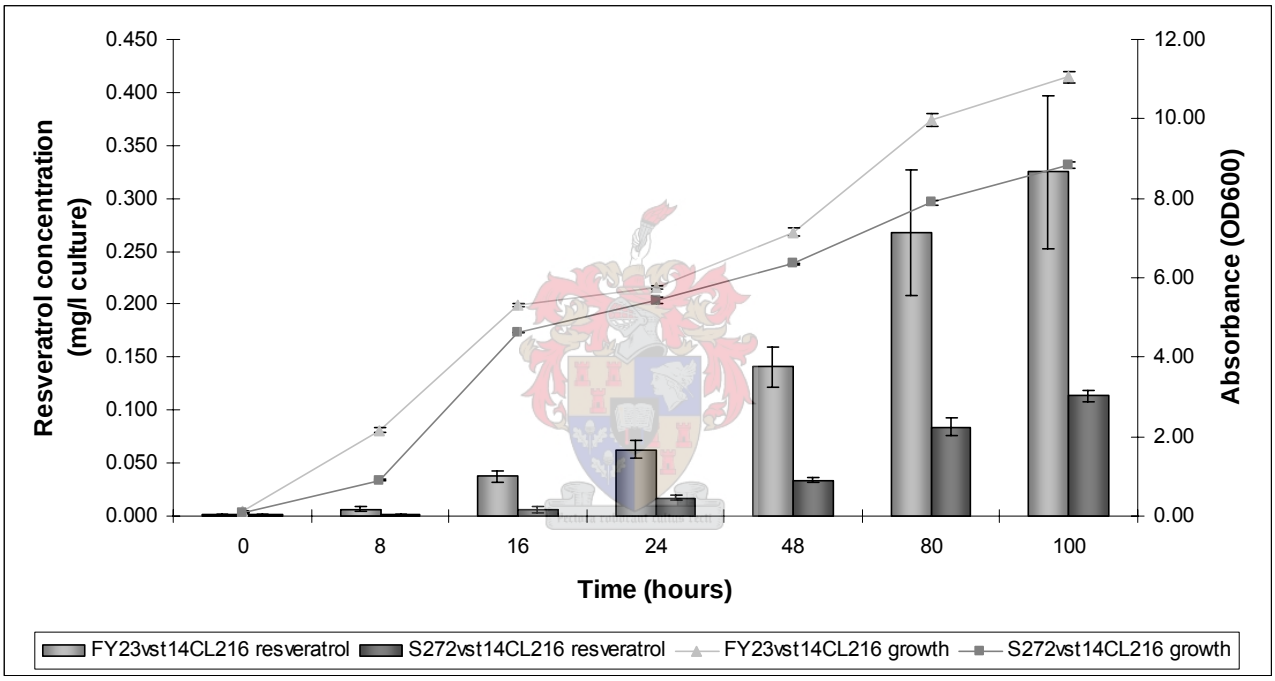


Figure 3.7. Time course resveratrol production by FY23 and Σ272 transformants cultured in rich media supplemented with 10 mg/l *p*-coumaric acid. Data are mean ± standard error (n = 5).

As a hypothetical exercise, resveratrol production was corrected for differences in growth between FY23 and Σ272 (**Figure 3.8**). Resveratrol production by Σ272 was adjusted to reflect levels that could have been produced, should it have achieved the same biomass production as FY23. The corrected values indicate that FY23 remains the superior resveratrol producer and this data supports our hypothesis that resveratrol production potential is dependent on yeast genetic background.

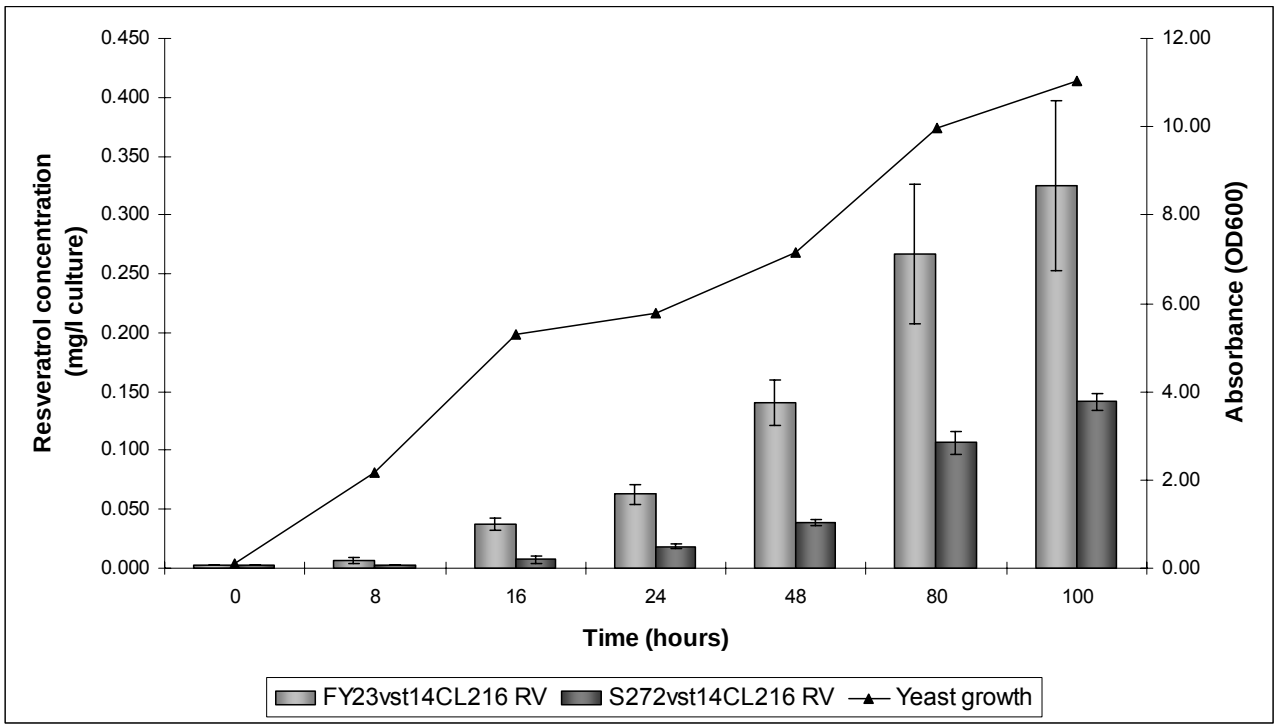


Figure 3.8. Time course resveratrol production by FY23 and Σ272 transformants, corrected for differences in biomass production. Data are mean ± standard error (n = 5).

The time period for analysis of resveratrol production in the CEN.PK2-1D genetic background was extended to 290 hours. This enabled us to determine when production peaked and to investigate the stability of resveratrol over an extended culture period. Resveratrol production steadily increased until 80 hours post inoculation, after which the levels remained stable throughout the experimental period (**Table 3.4**).

Table 3.4. Resveratrol production by CEN.PK2-1D transformants. Data are mean ± standard error (n = 5).

Time point	80 hours	100 hours	150 hours	290 hours*
Yeast growth (OD_{600})	8.6 ± 0.1	9.8 ± 0.1	10.8 ± 0.1	10.6 ± 0.2
[Resveratrol] (mg/l culture)	0.134 ± 0.032	0.158 ± 0.022	0.150 ± 0.015	0.160 ± 0.014

* (n = 4)

All of the results presented above confirm that genetic background does have an influence on resveratrol production by yeast transformed with *vst1* and *4CL216*. When comparing the high biomass producers, FY23 and CEN.PK2-1D, resveratrol was detected earlier in FY23 cultures than in CEN.PK2-1D cultures, where resveratrol was detected only 24 hours post inoculation. Σ272 began production later than FY23 but earlier than CEN.PK2-1D. The earliest time points where resveratrol was first detected in FY23 and Σ272 cultures corresponded to the exponential growth phase. However, resveratrol was

only detected once CEN.PK2-1D growth entered early stationary phase. A similar profile was observed by Beekwilder *et al*. (2006) for resveratrol production by the CEN.PK113-3b strain. As growth levelled off and reached stationary phase, resveratrol production continued to increase in FY23 and Σ272. This pattern of resveratrol production roughly mirrors metabolic activity in the yeast cell. Initially, no resveratrol was produced as transcription of the heterologous genes and protein production took place. As yeast biomass increased resveratrol production concomitantly increased. As was evidenced by the prolonged assay period for the CEN.PK2-1D strain, once resveratrol production had peaked, levels remained stable. It seems probable that equilibrium was reached between production and utilisation and/or degradation of resveratrol by the yeast.

3.3.2.6 Precursor feeding studies

The FY23 strain was selected for further experiments as it was the most efficient resveratrol producer. We investigated the effect of varying precursor concentrations on resveratrol yields. Firstly, we determined the effect of increasing the *p*-coumaric acid concentrations on yeast growth (**Figure 3.9**).

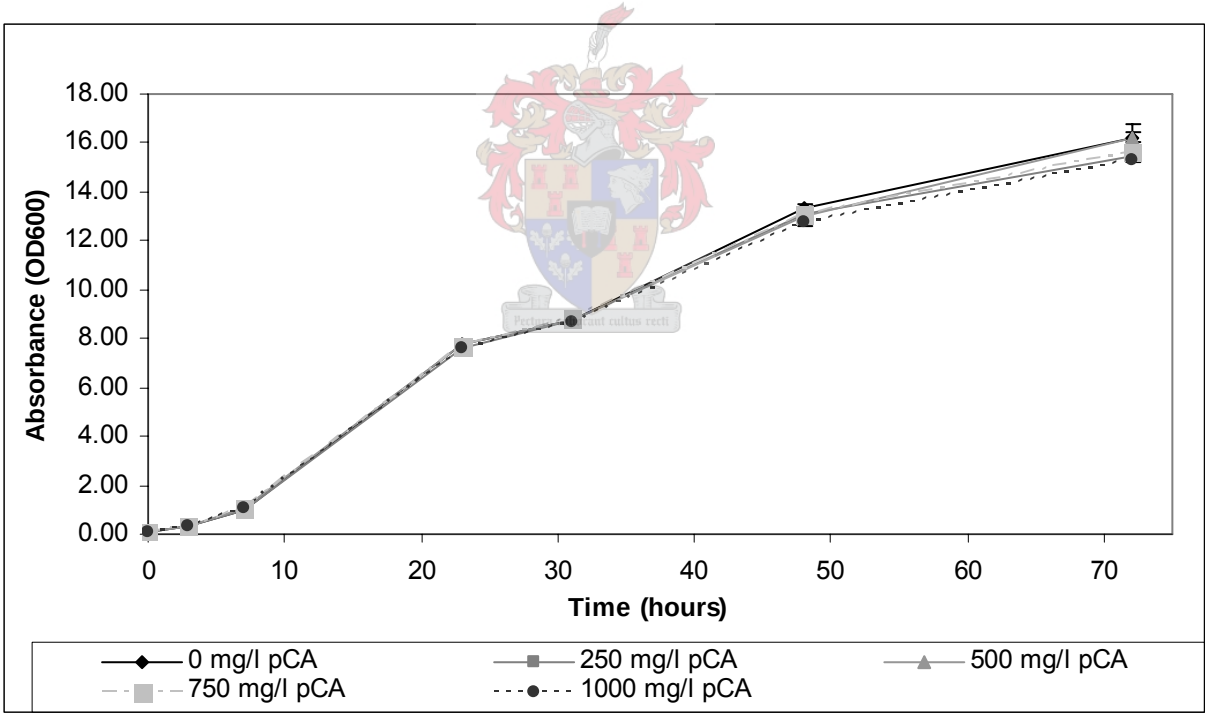


Figure 3.9. FY23 yeast growth in the presence of increasing concentrations of *p*-coumaric acid, a precursor for resveratrol biosynthesis. Data are mean + standard error (n = 4).

Yeast transformed with the control plasmids only was tested to ensure that any effect perceived could be attributed to the supplemented hydroxycinnamic acid rather than possibly resveratrol produced during growth. The figure shows that concentrations of up to 1000 mg/l *p*-coumaric acid did not affect yeast growth.

In previous experiments, the level of *p*-coumaric acid was kept constant at 10 mg/l. This level was initially selected for supplementation as it is similar to levels present in wine as reported in the literature. To our knowledge, values in grape must have not been reported. We increased *p*-coumaric acid concentrations up to tenfold those previously tested. In **Figure 3.10** results of resveratrol production by the FY23 yeast strain assayed after 48 hours of growth in increasing concentrations of *p*-coumaric acid are shown. Resveratrol production increased with an increasing supply of precursor.

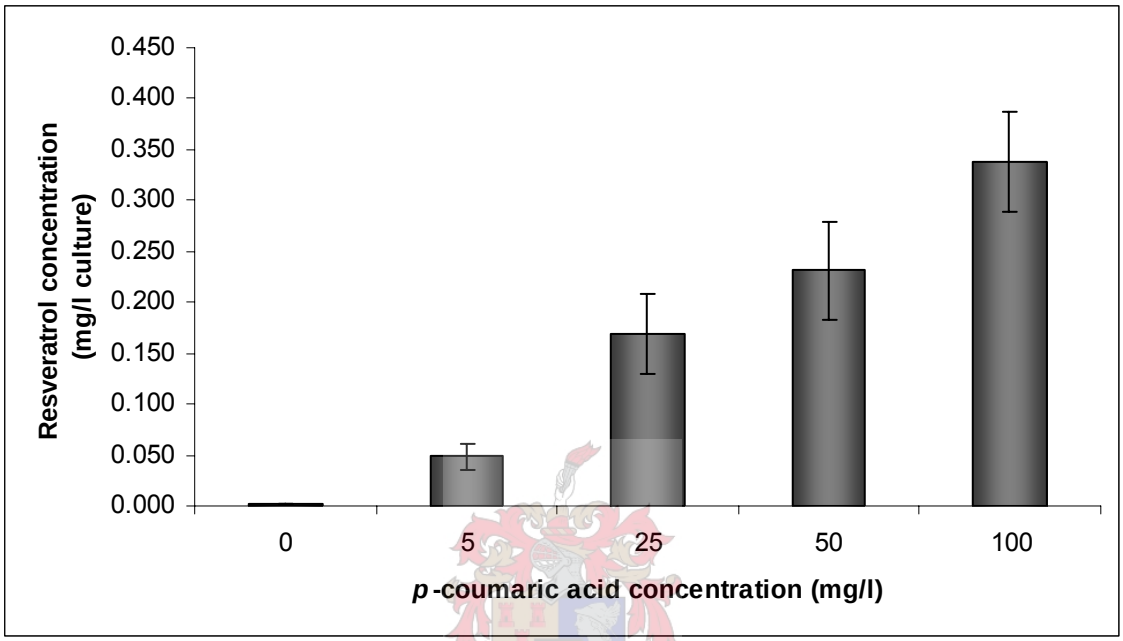


Figure 3.10. Resveratrol biosynthesis by FY23 yeast supplied with increasing levels of *p*-coumaric acid. Data are mean ± standard error (n = 5).

The trend observed in **Figure 3.10** indicated that the availability of *p*-coumaric acid seemed to be the limiting factor in resveratrol production under these experimental conditions. Should the levels of *p*-coumaric acid be further increased, it seems probable that a stage would be reached when the amount of malonyl CoA available for resveratrol synthesis would become limiting. As malonyl CoA also serves as a precursor for fatty acid biosynthesis the two pathways would, in all probability, eventually compete for substrate. It would be expected that the precursor would be channelled to the essential fatty acid biosynthetic pathway at the expense of resveratrol biosynthesis.

When compared to resveratrol production by the CEN.PK113-3b strain (Beekwilder *et al*., 2006), our results indicate that the amount of *p*-coumaric acid supplemented is an important factor to consider. After 48 hours of yeast growth, they reported levels of resveratrol in the culture media to be 3 mg/l in comparison to 0.35 mg/l in this study. However, they supplemented in excess of eight times the highest level of *p*-coumaric acid supplemented in this study. Precursor supplementation cannot be considered in isolation, as illustrated by comparing resveratrol production in *E. coli* (Beekwilder *et al*., 2006; Watts *et al.*, 2006). At similar time points, Beekwilder *et al*. measured 16 mg/l resveratrol in the

growth medium that was initially supplemented with 820 mg/l *p*-coumaric acid, while Watts *et al*. measured 105 mg/l resveratrol in the medium initially supplemented with 164 mg/l *p*-coumaric acid. The difference in the reported levels could not be ascribed to sample preparation methods prior to chemical analysis, as similar procedures were described by both groups. Different host strains, growth media and gene combinations were employed in the respective studies. These factors have been shown to influence resveratrol production in our work and further work done in our laboratory (not reported here).

Analyses of *p*-coumaric acid in the supernatant indicated that at all concentrations supplemented, there was between a 10 and 20% residual. The transport of *p*-coumaric acid into the cell may possibly account for this finding. As has been observed with the transport of other compounds into the cell, extracellular concentrations have an influence on the transport kinetics. *p*-Coumaric acid uptake by yeast has not been described in the literature and we hypothesise that it may enter the cell sharing the path of an unidentified compound. The possibility exists that the concentration gradient of *p*-coumaric acid influences the specificity of the transport into the cell. When the gradient is steep, transport becomes less specific and *p*-coumaric acid is transported into the cell. Conversely, when extracellular concentrations of *p*-coumaric acid are lower, the 'push' due to the concentration gradient is lessened and less precursor enters the cell. This may explain why not all of the precursor is taken up when lower concentrations of *p*-coumaric acid are supplemented. Clearly, further research is required in the field of transport of phenolic compounds by yeast.

In wine, *p*-coumaric acid occurs in its free form or as a tartaric acid ester. Levels of free *p*-coumaric acid have been reported to be between 5 and 8 mg/l in South African wines, which are higher than values reported for elsewhere in the world (de Villiers *et al.*, 2005). It is yet to be determined whether yeast would be able to utilise the bound form or if supplementation of free *p*-coumaric acid would be feasible to provide the yeast with optimal amounts of precursor for maximal resveratrol production. Hydroxycinnamic acids are known to be utilised by or affect other wine flora and the effect of supplementation on other wine parameters is not known. Alternately, yeast could be further engineered to produce *p*-coumaric acid. This has previously been accomplished by introducing genes encoding phenylalanine ammonia lyase (*PAL*) and cinnamate 4-hydroxlase (*C4H*) into *S. cerevisiae* (Ro and Douglas, 2004). In comparison, yeast harbouring only *PAL* from *Rhodosporidium toruloides* was shown to produce *p*-coumaric acid from tyrosine (Jiang *et al.*, 2005).

3.3.3 PHYSIOLOGICAL EFFECT OF RESVERATROL ON YEAST

It has been reported that exogenously supplied resveratrol increases yeast replicative lifespan (RLS) by up to 70% (Howitz *et al.*, 2003). It appears that endogenously produced resveratrol may have a similar effect. After an extended growth period of 290 hours, viable

cell numbers of non-producing yeast declined while resveratrol-producing yeast numbers remained stable (**Figure 3.11**).

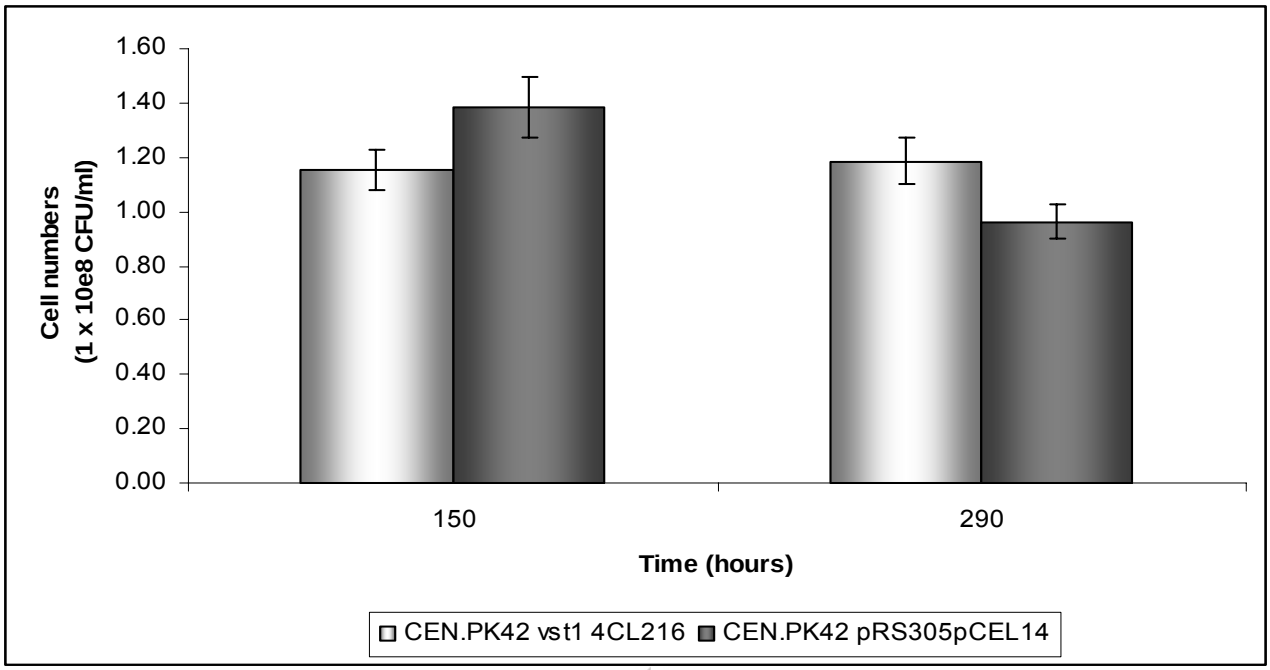


Figure 3.11. Comparison of yeast viability for resveratrol-producing (CEN.PK2-1Dvst14CL216) and non-producing yeast (CEN.PK2-1DpRS305pCEL14) cultivated for 290 hours. Data are mean ± standard error (n = 3).

Whether resveratrol influences replicative lifespan only or if it may also have an effect on chronological lifespan (CLS) is not known. It seems plausible, considering that resveratrol production begins within 24 hours, that there would be an effect on RLS. Howitz *et al.* (2003) found that resveratrol, at 2.3 mg/l or 23 mg/l, had no effect on CLS. **Figure 3.12** shows that endogenously produced resveratrol appears to have no effect on yeast cell morphology.

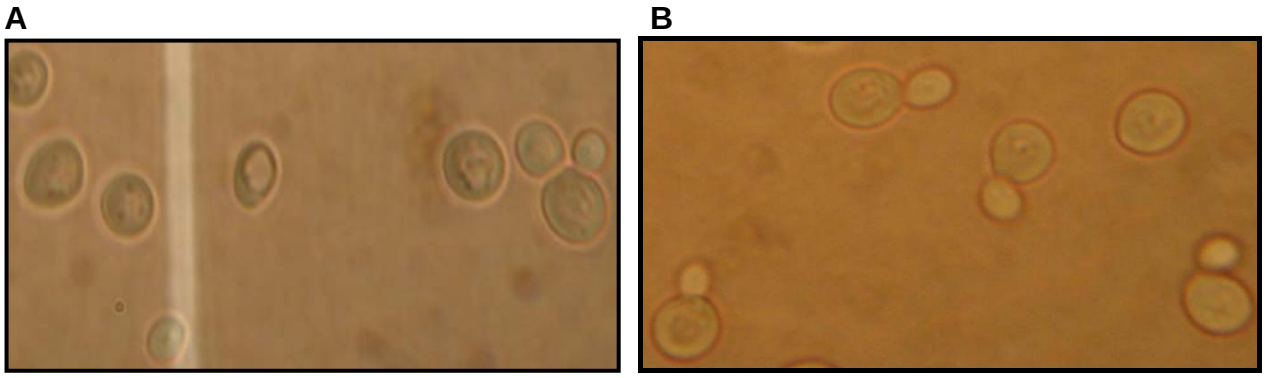


Figure 3.12. Yeast morphology of non-resveratrol-producing (A) and resveratrol-producing (B) yeast after 100 hours of growth in rich medium (1000x magnification).

Yeast morphology is typical of late stationary phase cultures with enlarged cells and when present, large buds that have remained attached to the mother cell. Viability staining

indicated no clear difference between resveratrol-producers and non-producers although it is not self-evident from **Figure 3.12**. Further work is required in order to confirm and clarify these preliminary results regarding the physiological effect of resveratrol on yeast lifespan. Yeast harbouring integrated copies of either *vst1* or *4CL216* only would be required as extra controls to confirm that yeast-produced resveratrol is indeed responsible for the observed effect.

3.4 ACKNOWLEDGEMENTS

We would like to thank Dr Sarita Groenewald for critical reading of the manuscript and the National Research Foundation (NRF), Winetech, THRIP and Stellenbosch University for financial support.

3.5 LITERATURE CITED

Adrian M, Jeandet P, Bessis R, Joubert JM. 1996. Induction of phytoalexin (resveratrol) synthesis in grapevine leaves treated with aluminum chloride ($AlCl_3$). *J Agric Food Chem* **44**: 1979-1981.

Arichi H, Kimura Y, Okuda H, Baba K, Kozawa M, Arichi S. 1982. Effects of stilbene components of the roots of *Polygonum cuspidatum* Sieb. et Zucc. on lipid metabolism. *Chem Pharm Bull* **30**: 1766-1770.

Ausubel FM, Brent R, Kingstin RE (eds). 1994. *Current Protocols in Molecular Biology*, vol. 13.0.1-13.14.17. Wiley: New York.

Becker JVW, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* **4**: 79-85.

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CHR, Bovy A. 2006. Production of resveratrol in recombinant microorganisms. *Appl Environ Microbiol* **72**: 5670-5672.

Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM. 2000. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology* **141**: 3657-3667.

Bradamante S, Barenghi L, Villa A. 2004. Cardiovascular protective effects of resveratrol. *Cardiovasc Drug Rev* **22**: 169-188.

Creasy LL, Coffee M. 1988. Phytoalexin production potential of grape berries. *J Am Soc Hortic Sci* **113**: 230-234.

de Villiers A, Majek P, Lynen F, Crouch A, Lauer H, Sandra P. 2005. Classification of South African red and white wines according to grape variety based on the non-coloured phenolic content. *Eur Food Res Technol* **221**: 520-528.

Delmas D, Jannin B, Latruffe N. 2005. Resveratrol: preventing properties against vascular alterations and ageing. *Mol Nutr Food Res* **49**: 377-395.

Dercks W, Creasy LL. 1989. Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. *Physiol Mol Plant Pathol* **34**: 203-213.

Dong Z. 2003. Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat Res* **523-524**: 145-150.

Entian KD, Kötter P. 1998. Yeast mutant and plasmid collections. In *Methods in Microbiology*, 26, Brown JPA and Tuite MF (eds). Academic Press: San Diego; 431-449.

Fauconneau B, Waffo-Teguo P, Huguet F, Barrier L, Decendit A, Merillon JM. 1997. Comparative study of radical scavenger and antioxidant properties of phenolic compounds from *Vitis vinifera* cell cultures using *in vitro* tests. *Life Sci* **61**: 2103-2110.

Frankel EN, Waterhouse AL, Kinsella JE. 1993. Inhibition of human LDL oxidation by resveratrol. *Lancet* **341**: 1103-1104.

Gamoh K, Nakashima K. 1999. Liquid chromatography/mass spectrometric determination of *trans*-resveratrol in wine using a tandem solid-phase extraction method. *Rapid Commun Mass Spectrom* **13**: 1112-1115.

Gehm BD, McAndrews JM, Chien PY, Jameson JL. 1997. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc Natl Acad Sci USA* **94**: 14138-14143.

Gietz RD, Schiestl RH. 1995. Transforming yeast with DNA. *Methods Mol Cell Biol* **5**: 255-269.

Goldberg DM, Yan J, Ng E, Diamandis EP, Karumanchiri A, Soleas G, Waterhouse AL. 1995. A global survey of *trans*-resveratrol concentrations in commercial wines. *Am J Enol Vitic* **46**: 159-165.

Gundllapalli SB, Cordero Otero RR, Pretorius IS. 2006. Development of a screening method for the identification of a novel *Saccharomyces cerevisiae* mutant over-expressing *Trichoderma reesei* cellobiohydrolase II. *Ann Microbiol* **56**: 95-102.

Hain R, Reif H-J, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stocker RH, Stenzel K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361**: 153-156.

Hao HD, He LR. 2004. Mechanisms of cardiovascular protection by resveratrol. *J Med Food* **7**: 290-298.

Higuchi R, Krummel B, Saiki RK. 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucl Acids Res* **16**: 7351-7367.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* **425**: 191-196.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**: 218-220.

Jiang H, Wood KV, Morgan JA. 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 2962-2969.

Kimura Y, Okuda H, Arichi S. 1985. Effects of stilbenes on arachidonate metabolism in leukocytes. *Biochim Biophys Acta* **834**: 275-278.

Langcake P, Pryce RJ. 1976. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiol Plant Pathol* **9**: 77-86.

Langcake P, Pryce RJ. 1977. The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. *Phytochemistry* **16**: 1193-1196.

McMurtrey KD, Minn J, Pobanz K, Schultz TP. 1994. Analysis of wines for resveratrol using direct injection high-pressure liquid chromatography with electrochemical detection. *J Agric Food Chem* **42**: 2077-2080.

Olas B, Wachowicz B. 2005. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. *Platelets* **16**: 251-260.

Pezet R, Pont V, Cuenat P. 1994. Method to determine resveratrol and pterostilbene in grape berries and wines using high-performance liquid chromatography and highly sensitive fluorimetric detection. *J Chromatogr, A* **663**: 191-197.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339**: 1523-1526.

Ro D-K, Douglas CJ. 2004. Reconstitution of the entry point of plant phenylpropanoid metabolism in yeast (*Saccharomyces cerevisiae*): IMPLICATIONS FOR CONTROL OF METABOLIC FLUX INTO THE PHENYLPROPANOID PATHWAY. *J Biol Chem* **279**: 2600-2607.

Romero-Perez AI, Lamuela-Raventos RM, Andres-Lacueva C, de la Torre-Boronat MC. 2001. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J Agric Food Chem* **49**: 210-215.

Rose MD, Winston F, Hieter P. 1990. *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Sambrook J, Fritsch EF, Maniatis T (eds). 1989. *Molecular cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Siemann EH, Creasy LL. 1992. Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic* **43**: 49-52.

Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19-27.

Trela BC, Waterhouse AL. 1996. Resveratrol: Isomeric molar absorptivities and stability. *J Agric Food Chem* **44**: 1253-1257.

Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A. 2006. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr Biol* **16**: 296-300.

Watts KT, Lee PC, Schmidt-Dannert C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol* **6**: 22-34.

Winston F, Dollard C, Ricupero-Hovasse SL. 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11**: 53-55.

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D. 2004. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**: 686-689.

Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG, Huang YZ. 2001. Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review). *Int J Mol Med* **8**: 3-17.

GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION AND CONCLUSIONS

Subsequent to a broadcast on American television where the initial findings of the French paradox were revealed to the general public, red wine sales increased by 39% in the year following the broadcast (Sherwood, 1993). Although all the factors responsible for the phenomenon were not clear, the public response was nevertheless substantial. The willingness of consumers to accept products that will provide added health benefits cannot, and has not, been overlooked from the marketing perspective. The global overproduction of wine has translated into a highly competitive market, with competition ever-increasing due to quality wines continually entering the market from new world wine producers. Being able to supply a unique and improved product would confer a competitive advantage to the producer.

To this end, our research is focused on the production of a wine yeast strain capable of producing resveratrol during fermentation of grape must. Ultimately, being able to claim that added health benefits may be derived from consumption of the product should confer a unique selling point to wines produced with the resveratrol-producing strain. The approval of the first genetically modified (GM) wine yeast by the US Food and Drug Administration in 2003 may pave the way for the products of our research regarding critical consumer attitudes towards the use of GM yeast for the production of wine.

Initial work by Becker *et al.* (2003) together with work done in this follow-up study have evaluated the requirements for resveratrol production by *S. cerevisiae* laboratory strains. In order to effectively evaluate resveratrol production by yeast, the analytical tools required optimisation. We optimised the extraction of resveratrol from the intracellular fraction of yeast and also devised a procedure for the extraction from the extracellular fraction. The LCMSMS method was further developed to identify and quantify other compounds which were associated with evaluating resveratrol production in yeast. Subsequent to the optimisation of the expression of the genes required for resveratrol synthesis, we were able to evaluate resveratrol production in different growth media. Interestingly, we found that yeast accumulated resveratrol intracellularly when cultivated in minimal media, but no resveratrol was detected in the intracellular extracts when yeast was cultivated in rich media. Resveratrol was only detected in the extracellular fraction. The reason for this observation remains unexplained, yet highlights the variability of yeast functioning under different growth conditions. That yeast was able to secrete a metabolite that it naturally is unable to produce, is also remarkable.

Yeast genetic background was investigated as a factor that could influence the production of resveratrol by yeast. It was shown to influence both production kinetics and resveratrol yield. When comparing the two high biomass producing strains FY23 and

CEN.PK2-1D, resveratrol production commenced earlier in FY23 and the resveratrol yield was significantly higher. FY23 production peaked at 0.325 mg/l while CEN.PK2-1D produced 0.158 mg/l. The low biomass producing strain, Σ272, fell short of both the higher biomass producers with regards to resveratrol production – levels peaked at 0.113 mg/l. These findings highlight the importance of selecting the best possible wine yeast strain to transform. The logistics of doing so however, are somewhat challenging, possibly even unfeasible. It would in all probability be on a trial-and-error basis unless the basis for the differences in resveratrol production potential was to be elucidated. Within a specific genetic background, the inherent capacity of different individuals to produce resveratrol was also highly variable. In addition to genetically enhancing a wine yeast strain to produce resveratrol, selection of superior individual producers may also be a strategy to obtain maximal resveratrol yields.

Precursor feeding studies indicated that the availability of *p*-coumaric acid was limiting. Levels in grape must have not been determined but in wine they are equivalent to the lower levels of *p*-coumaric we supplemented. It would therefore be necessary to either supplement *p*-coumaric acid or engineer the yeast to produce its own *p*-coumaric acid to maximise resveratrol production. The factors influencing resveratrol production by recombinant organisms cannot be considered in isolation. When comparing the yields of resveratrol obtained in *Escherichia coli* (Beekwilder *et al.*, 2006; Watts *et al.*, 2006), Watts *et al*. obtained five fold more resveratrol than Beekwilder *et al*., yet they supplemented a fifth of the level of *p*-coumaric acid supplemented by Beekwilder *et al*. Differences could be attributed to either growth medium, host strain, the combination of genes expressed or possibly a combination of the aforementioned factors. Genes encoding resveratrol synthase and coenzyme A ligase have been isolated from a range of plants. The optimal combination of genes from different plant sources, expressed in a recombinant host, could thus also be a factor affecting resveratrol production. The results obtained in *E. coli* indicate that it may be a possibility, although no exclusive experimental data confirm this. Work in our laboratory (not reported in this study) provides confirmation – expressing coenzyme A ligase from tobacco with resveratrol synthase from grapevine improves resveratrol yields when compared to the coenzyme A ligase from hybrid poplar and resveratrol synthase from grapevine combination. Furthermore, selection of the optimal combination of genes would need to be investigated in order to obtain maximal metabolite production, yet limit by-product formation as observed by Jiang *et al*. (2005).

Optimisation of resveratrol production in this study resulted in an increase in resveratrol production from 1.45 μg/l (as measured by Becker *et al*., 2003) to 0.35 mg/l. Beekwilder *et al*., (2006) reported production of resveratrol at 5.8 mg/l in *S. cerevisiae*. Should the optimal growth period and precursor levels be selected, similar levels could probably be attained in our system.

On a more fundamental level, the effect of resveratrol on yeast physiology merits further study. Our results show that resveratrol-producing strains maintained their viability over an extended culture period while the non-producing strains showed signs of

decreased viability. Further elucidation of the observation would contribute to the understanding of the effect resveratrol has on yeast, if any, as to date no consensus has been reached (Howitz *et al.*, 2003; Kaeberlein *et al.*, 2005).

In conclusion, this study builds on and forms part of the vital groundwork required to effectively engineer a wine yeast strain for maximal resveratrol production.

4.2 LITERATURE CITED

Becker JVW, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* **4**: 79-85.

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CHR, Bovy A. 2006. Production of resveratrol in recombinant microorganisms. *Appl Environ Microbiol* **72**: 5670-5672.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* **425**: 191-196.

Jiang H, Wood KV, Morgan JA. 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 2962-2969.

Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A, Kennedy BK. 2005. Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem* **280**: 17038-17045.

Sherwood, B. 1993. Wine and Poses. *Washington Monthly*.

US Food and Drug Administration Centre for Food Safety and Applied Nutrition (CFSAN) Office of Food Additive Safety Agency Response Letter. GRAS Notice No. 000120. June 30, 2003

Watts KT, Lee PC, Schmidt-Dannert C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol* **6**: 22-34.